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MECHANISTIC INSIGHT INTO GRAVITROPIC RESPONSES OF ARABIDOPSIS HYPOCOTYL

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Abbreviations

ABCB: B subclass of ATP-binding cassette

ABP1: AUXIN BINDING PROTEIN1

ABP1 AS: *abp1* antisense line

ACT: ACTIN

AFB: AUXIN-RELATED F-Box protein

ARF: AUXIN RESPONSE FACTOR

ARF: ADP-ribosylation factor

ARF GTPase-activating proteins

AUX1: AUXIN RESISTANT1

Aux/IAA: auxin/indole-3-acetic acid

AUX/LAX: AUXIN/LIKE AUXIN

BFA: brefeldin A

BEN: BFA-visualized endocytic trafficking defective

BEX: BFA-visualized exocytic trafficking defective

CHX: cycloheximide

CW: cell wall

D6K: D6 PROTEIN KINASE

DMSO: dimethyl sulfoxide

EE: early endosome

EMS: ethyl methane sulfonate

ER: endoplasmic reticulum

GAP: ARF GTPase-activating protein

GEF: guanine-nucleotide exchange factors

GNL1: GNOM-LIKE1

GFP: green fluorescent protein

GUS: β-glucuronidase

IAA: indole-3-acetic acid

LatB: latrunculin B

LR: lateral roots

LRP: lateral root primordium

MFs: actin microfilaments

MIN7: ARF GEF BEN1/HOPM Interactor 7

MT: microtubule

MUNC18: SEC1-/mammalian uncoordinated-18

NAA: naphthalene-1-acetic acid

NGS: Next-Generation Sequencing

PEO-IAA: a-(phenyl ethyl-2-one)-indole-3-acetic acid

PGP: P-glycoprotein

PHOT: PHOTOTROPIN

PID: PINOID

PIN: PIN-FORMED

PM: plasma membrane

PP2A: protein phosphatase 2A

Q-RT-PCR: semi-quantitative reverse transcriptase polymerase chain reaction

R:FR: red and far-red light

RabA1b: Ras genes from rat brainA1b

RFP: red fluorescent protein

SCR: SCARECROW

TIR1: TRANSPORT INHIBITOR RESPONSE1

TGN: trans-Golgi network

Tyrph: Tyrphostin

WM: wortmanin

WT: wild type

2,4-D: 2,4-dichlorophenoxy acetic acid

5-F-IAA: 5-fluoroindole-3-acetic acid

Scope

As sessile organisms, plants adapt their growth and development to respond to fluctuating environmental conditions. Plant cells perceive endogenous and exogenous stimuli, integrate them, and react to them to adapt their development. In the 19th century, Darwin (1880) predicted the existence of substances that regulated the coordinated plant growth. Some decades later, Went (1937) identified these growth substances as small signaling molecules, designated plant hormones or phytohormones. Over the past decades, studies that focused on the mechanisms of phytohormone regulation of plant development clearly demonstrated that the individual hormonal action is largely determined by complex crosstalk interactions with other hormonal signaling pathways. Plant hormones, including auxin, cytokinin, ethylene, gibberellin, brassinosteroids, strigolactone, jasmonic acid, and abscisic acid, control a number of developmental processes, including embryogenesis, root and shoot growth and branching, flowering, but also growth responses to light or gravity (Vanstraelen & Benková, 2012). Although the main principles of signal perception and transduction for most plant hormones have been characterized, the molecular components for hormonal signal integration remain largely unknown. Considering the complexity of the biology of phytohormones, we centered our research on the molecular mechanism underlying the role of auxin. The focus is on the investigation of the molecular mechanisms of auxin transport, accumulation, and signaling by means of young *Arabidopsis thaliana* seedlings (root and hypocotyl) as working model system.

In plants, changes in gravity direction are recognized in specialized heavy starch amyloplast-containing cells (statocysts) that sediment to the cell bottoms (Hashiguchi et al, 2013). This signal is translated into an asymmetric auxin distribution in responsive organs (root tips and hypocotyls/stems). The most straightforward explanation for changes in the auxin flow is the modification of the polar subcellular localization of auxin transporters (Friml et al., 2002). The PIN-FORMED3 (PIN3) auxin efflux carrier, the main candidate for these responses, localizes at the plasma membrane (PM) of columella root cells and hypocotyl endodermal cells. The resulting auxin accumulation at the lower side inhibits in roots, or stimulates in hypocotyls, cell elongation, respectively, with organ bending as a consequence (Kleine-Vehn et al, 2010; Rakusová et al, 2011). Moreover, bending of etiolated hypocotyls in response to gravity stimulation is a dynamic and regulated process. With time-lapse experiments, we observed that the bending response, i.e. the asymmetric cell elongation, is terminated when a certain angle is reached, followed by a straight elongation, avoiding overbending. The regulated auxin flow,

necessary for proper plant development and for adapted growth to light and gravity, was the main subject of the study presented here.

The introductory **Chapter 1** gives an overwiew of the current knowledge on auxin role during plant development and responses to environmental changes with the focus on tropic responses of hypocotyl. **Chapter 2** illustrates how the regulation of the PIN3 polarity controls the redistribution of auxin in response to gravity changes in hypocotyls. **Chapter 3** describes the cellular mechanisms of the PIN3-dependent regulation of this hypocotyl bending response. We detected dynamic changes in PIN3 polarity and asymmetric auxin distribution during the gravity response. To discover new regulators of PIN3 polarization during tropic responses, a forward genetic screen was done with gravitropic bending as phenotype to select mutants potentially affected in the PIN3 polarity machinery. In **Chapter 4** the screen and two the most interesting candidates are presented in detail. **Chapter 5** describes how auxin feedback regulates the cellular polarity of its own transporters, namely the PIN1 and PIN2 efflux carriers, thereby determining the directionality of its own stream during plant development (Sauer et al, 2006).

All together, the data presented in this PhD thesis aim to bring new insights into the PIN polar targeting that result in the (re)establishment of the auxin flow and accumulation during plant development and environmental responses.

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Chapter 1.

Introduction

Adapted from

***Rakusová, H**., Fendrych M., Friml, J. Intracellular trafficking and PIN-mediated cell polarity during tropic responses in plants. (Submitted to Current Opinion in Plant Biology)*

HR wrote the manuscript and JF and MF assisted manuscript writing.

Intracellular trafficking and PIN-mediated cell polarity during plant development and tropic responses

Auxin controls plant growth and development

Plant growth and development is controlled by plant hormones (phytohormones) which are present in very low concentrations. The phytohormones include indole-3-acetic acid (IAA) or auxin, abscisic acid, brassinosteroids, cytokinin, gibberellin, jasmonic acid, salicylic acid, ethylene and strigolactone. The plant architecture and various developmental processes in plants are largely dependent on the coordinated activity of one of them, a small signalling molecule - auxin. Auxin plays a major role in management of many growth and behavioural processes during the plant's life cycle. The dynamic pattern of the auxin distribution within the plant body regulates development and responses to the external stimuli. Examples in which auxin regulate plant development are appropriate embryo development, post-embryonic growth involving the activity of meristem tissues (Figure 1). All these processes require a differential distribution of auxin (Petrášek and Friml, 2009). Several factors, such as auxin biosynthesis, conjugation, cell compartmentalization, degradation, and transport can modify the free auxin levels (reviewed in Taiz and Zeiger, 2006; Ljung, 2013). The highest auxin biosynthesis was detected in shoot meristematic tissues and root, in young cotyledons and leaves where the main biosynthesis capacity in older plant stages is located (Ljung et al., 2005; 2013). Auxin transport is than regulated in two different manners – (i) quick and passive non-polar auxin transport via phloemfor long-distance transport and (ii) slow, regulated intracellular transport (Robert and Friml et al., 2009). The proper auxin distribution is achieved through the well-coordinated active transport of auxin molecules from cell to cell by the so-called polar auxin transport.

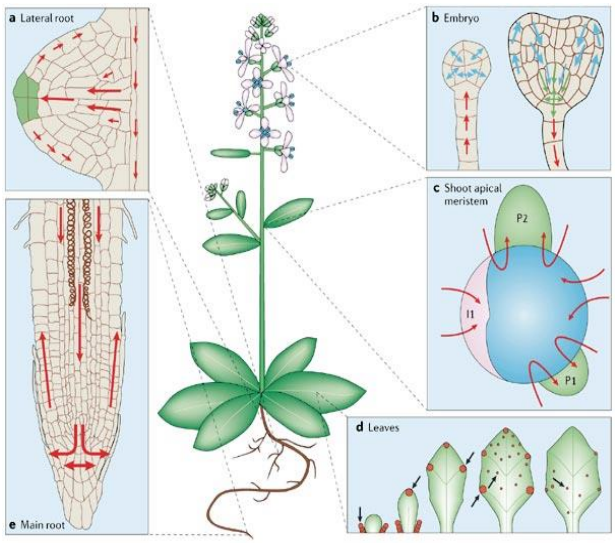

Figure 1. Auxin flow and accumulation during developmental stages in *Arabidopsis thaliana*.

(a) Directional auxin flow results in an auxin maximum in the lateral root tip and promotes outgrowth. Auxin is indicated in green and auxin transport by red arrows. (b) Auxin is provided to the young-stage embryo and the auxin flux is reversed at later stages, leading auxin out of the embryo. The transport direction by PIN proteins is indicated by arrows. (c) In the shoot apical meristem, auxin is redirected towards the site of new leaf formation. The shoot apex is indicated in blue. (d) Vascular development and leaf shape patterning are mediated by auxin. Arrows indicate auxin biosynthesis and the red dots the auxin maxima. (e) PIN proteins determine the flux of auxin towards the root tip in the stele, and back in the epidermis. This movement allows quick responses to gravity. Auxin transport is indicated by red arrows. (*source: Teale et al., 2006*)

The classical chemi-osmotic model postulated a transmembrane auxin transporter (Rubery & Sheldrake, 1974). The auxin flow directionality within tissues is then determined by a cellular, polar localization of these transporters - the efflux auxin carriers, such as the B subclass of ATP-binding cassette (ABCB), the P-glycoproteins (PGPs) (Geisler & Murphy, 2006) and PIN-FORMED (PIN) proteins (Petrášek et al, 2006) with the help of the influx auxin carriers, such as the AUXIN/LIKE AUXIN (AUX/LAX) (Band et al, 2014). The polar cellular location of the PIN proteins has been shown to direct the auxin flow (Wiśniewska et al, 2006).

PIN polarity regulates directional auxin transport during plant development

Polarities of individual cells and of tissues are closely connected by the auxin flow (Sauer et al. 2006). Cellular biological processes that depend on vesicle trafficking and polar targeting have an auxin-mediated developmental output. Individual plant cells polarize in a coordinated manner to integrate the polarizing signal into the tissue context. In the canalization model (Sachs, 1981), auxin itself acts as a polarizing cue and feeds back on the intracellular auxin flow to synchronize polarity rearrangements (Sauer et al, 2006). A mechanistic conception for the canalization model has been proposed (Wabnik et al., 2010) in which the intracellular auxin signaling for the PIN expression and the theoretical extracellular auxin signaling for PIN modulation are combined for the PIN subcellular dynamics. A computer model recapitulated the experimentally observed patterns of tissue polarization followed by an asymmetric auxin distribution during the leaf vascular formation. PIN polarization and canalization of the auxin flow in plants are relevant in various developmental stages. Indeed, plants have the ability to redefine cell and tissue polarities in different developmental processes, such as embryogenesis (Friml et al., 2003; Wabnik et al., 2013), postembryonic organogenesis, such as lateral root formation (Benková et al., 2003; Geldner et al., 2004; Marhavý et al., 2013), or vascular tissue formation (Sauer et al., 2006; Scarpella et al., 2006). During these developmental changes, auxin accumulation often varies by the PIN polarity reestablishment. Such a repolarization process, called transcytosis, represents the dynamic translocation of the proteins from one distinct plasma membrane (PM) domain to another via intracellular trafficking (Kleine-Vehn et al., 2008a; 2008b).

Auxin is essential throughour the full plant life, it is implicated since the first axis formation during embryogenesis. For instance, during embryonic development rapid PIN relocations have been represented by PIN1 and PIN7 that undergo a polarity switch at the globular stage (Friml et al., 2003; Wabnik et al., 2013). PIN7 is localized apically in the

suspensor and PIN1 is mostly nonpolar in the proembryo, whereas later on PIN1 polarizes to the basal side of cells adjacent to the future root meristem. PIN7 changes its polarity from apical to basal (Friml et al. 2003). These PIN polarity alterations subsequently lead to the rearrangement of auxin accumulations at the presumptive embryo root pole and are the necessary factors for root specification during embryogenesis (Friml et al., 2003; Wabnik et al., 2013).

During the postembryonic development, PIN polarity changes are observed while lateral root primordia are formed. Polarization of PIN3 in endodermis cells helps auxin to flow from the external cell layer to the pericycle and contributes to the peak of auxin response that leads to founder cell division (Marhavý et al., 2013). Then, PIN1 localization during lateral root primordia development changes from anticlinal towards periclinal cell sides, pointing to the presumptive primordium tips. These variations result in the establishment of a new auxin maximum at the primordium tip and stimulate lateral root formation and outgrowth (Benková et al., 2003; Geldner et al., 2004).

Vascular tissue formation during leaf venation (Scarpella et al., 2006) is another example of auxin-dependent reorganization of plant tissues. Cells perceive their position within the tissue and recognize their orientation relative to the remainder of the leaf cells. Here, by a positive feedback mechanism, auxin induces the capacity and polarity of its own transport, gradually rearranging cell polarity and repolarization of neighbouring cells. The canalization hypothesis (Sachs, 1981) proposes such a feedback effect of auxin on the directionality of the auxin intercellular flow (Sauer et al., 2006), resulting in vascular stream establishment.

In all these instances, changes in the PIN polarity are followed by the redirection of auxin fluxes and the rearrangement of local auxin accumulation patterns (auxin gradients) that trigger changes in developmental programs (reviewed in Kleine-Vehn and Friml, 2008). Taken together, developmental outputs, but also auxin itself, act as polarizing cues that link individual cell polarity with tissue and organ polarities through the control of the PIN polar targeting.

Tropism in plants

Plants and animals respond differently to environmental stimuli and to competitors. Whereas animals can react with a behavioural response manifested by run or fight, plants, as sessile organisms, display an adaptive and flexible development that optimally adjusts the growth to the environment. Plants have evolved sophisticated mechanisms to perceive information from their biotic and abiotic surroundings. Appropriate post-embryonic growth,

involving the activity of meristem tissues, and tropic growth responses are examples, in which plants adapt to environmental conditions. Tropism is a directional growth of a plant in response to an environmental stimulus, such as light (phototropism) and gravity (gravitropism). Phototropism enables plants to react to changes in light direction by bending towards a unidirectional light source, essential for photosynthetic plants. Gravitropism is the growth reaction that orients plant development along a gravity vector. Phototropism and gravitropism involve light or gravity perception and the asymmetric distribution of the phytohormone auxin (Went and Thimann, 1937). Auxin induces hypocotyl cell elongation. Subsequent elongation of the cells on the shaded/lower side of shoots results in differential growth and bending towards the light source or against the gravity vector. In case of roots, the asymmetrical auxin response is the same, but the organ reacts oppositely.

The response of hypocotyls growing in the soil to reach the light is not often described and is of high importance for the proper plant development. Results obtained the last years, support the observations that auxin and polarization of PIN proteins play an important role in plant phototropism and gravitropism (Ding et al., 2011; Kleine-Vehn et al., 2010; Rakusová et al., 2011).

PIN CELLULAR (RE)POLARIZATION DURING TROPISM

Phototropism

Plants sense the intensity, direction, duration, and wavelength of light (for a recent review, see Liscum et al., 2014). Light sensing occurs in the upper hypocotyl (Preuten et al., 2013) where also bending takes place. The blue-light receptors, such as PHOTOTROPIN1 (PHOT1) and PHOT2 (Huala et al., 1997) that combine blue-light sensing with kinase activity, can initiate the phototropic response with shoot bending as a consequence. One of the PHOT1 phosphorylation targets is the auxin transporter ABCB19 (Christie et al., 2011) that, in the absence of light, has been proposed to mediate the auxin flux through the vasculature. Following blue-light radiation, auxin is redistributed from the vasculature to the epidermis in the upper part of the hypocotyl by the PHOT1-mediated phospohorylation that inhibits the ABCB19 activity. Subsequently, auxin is channelled by PIN3 through the epidermis to the elongation zone. Alternatively, PIN3 has been shown to play a role during phototropism in endodermal cells and to mediate lateral auxin fluxes by restricting auxin to the vascular cylinder (Ding et al., 2011). The expression of PIN3 in endodermal cells has an apolar localization in dark-grown hypocotyls. After the unidirectional light stimulation, PIN3 relocates from the outer

side of endodermal cells at the illuminated hypocotyl side to the inner cell side and prompts the auxin flow to the shaded side of hypocotyls, shown by increased DR5 auxin reporter expression (Ding et al., 2011). Consequently, auxin accumulation induces cell elongation at the shaded side, resulting in hypocotyl bending (Ding et al., 2011).

The role of the PIN proteins in phototropism has been characterized under various light conditions and by means of different mutant alleles (Haga and Sakai, 2013). The *pin3* alleles, presumably not full knock-out (such as *pin3-3*), have no altered phototropic response (Haga et al., 2012; 2013; Willige 2013), whereas other alleles have a clear effect (for example *pin3-4*) (Ding et al., 2011; Rakusová et al., 2011). The weak phenotype of the *pin3* mutants is due to the PIN3, PIN4, and PIN7 redundancy during phototropism (Ding et al., 2011; Haga and Sakai, 2012, 2013; Willige et al., 2013). Finally, phototropism can be completely inhibited by the treatment with NPA (Friml et al., 2002), known to block auxin transport, but by a not-completely-understood mechanism. Taken together, the complete inhibition of phototropism by chemical prevention of the polar auxin transport is more effective, because it influences the auxin transport more globally than genetic manipulation.

Gravitropism

In the hypocotyls of plants, gravity is sensed by the sedimentation of amyloplasts in endodermal cells (Hashiguchi et al., 2013). One model postulates that the gravity signal transduction starts when sedimenting amyloplasts promote the opening of mechano-sensitive ion channels, either directly or through interaction with the actin cytoskeleton. Alternatively, signal transduction may initiate after sedimentation, when amyloplast-borne ligands interact with receptors located on sensitive structures within the statocytes (reviewed in Hashiguchi et al., 2013).

The gravitropic response is well described in *Arabidopsis thaliana* roots, where the auxin transport is mediated via auxin efflux and influx carriers, namely PIN1, PIN2, PIN3, PIN7, and AUX1. Starch-containing cells in the columella root cap sense the directional changes in gravity. Following gravi-stimulation, the heavy starch-containing amyloplasts sediment in columella cells (Band et al., 2012; Friml and Palme, 2002; Harrison and Masson, 2008; Morita, 2010), PIN3 and PIN7 re-localize from their originally uniform distribution to the side according to the gravity vector to promote a lateral auxin flow towards the lower side of the gravi-stimulated root (Friml et al., 2002; Harrison and Masson, 2008; Kleine-Vehn et al., 2010) with the help of PIN2 (Luschnig et al., 1998) and AUX1 (Bennett et al., 1996; Yang et al., 2006) and translocate auxin to the elongation zone. As a consequence, the auxin flow is

redirected to the lower side of the root, where it accumulates and inhibits growth, resulting in asymmetrical growth and gravitropic root bending.

The main player in the differential auxin transport during hypocotyl gravitropism is PIN3, the mutant of which shows no differential auxin distribution and reduced bending response (Friml et al., 2002). After gravity has been sensed by the amyloplasts sedimentation are relocated in endodermal cells, the PM localized PIN3 re-locates to the lower side of cells, presumably redirecting the auxin flow (Rakusová et al., 2011) (Figure 1). Whether the ABCB-mediated auxin transport plays a role also during gravitropism is an open question.

Shade avoidance

Plants have to compete for light and nutrients and display typical responses to the presence of neighbouring plants. One type of these behaviours is shade avoidance (Novoplansky, 2009) that, strictly speaking, is not a tropic response, because it leads to a uniform organ elongation. The phytochrome receptor signaling pathway allows plants to monitor the ratio between red and far-red light (R:FR) that is specific for shade caused by neighbouring plants (Ballare, 1999). In the shade of neighbours, the ratio shifts to far-red, so that perception of low R:FR ratios initiates shade avoidance responses (Ballare et al., 1990; Pierik and de Wit, 2014). Here, the *PIN3* expression is induced by the low R:FR ratio and in the hypocotyl endodermal cells PIN3 relocate from a basal to an outer-lateral localization, triggering an auxin efflux into the outer cell layers of the hypocotyl. Consequently, the hypocotyl elongates, exerting the typical shade avoidance response (Keuskamp et al., 2010b).

Either chemical inhibition or genetic interference with the polar auxin transport in *pin* mutants restrict shade avoidance-induced elongation (Keuskamp et al., 2010a). Relatively weak effects of the single *pin* mutations again imply functional redundancy and/or involvement of the ABCB auxin transporters. The connection between light-sensing responses (phototropism and photomorphogenesis), shade avoidance, and specifically, the role and regulation of PIN3 in both responses is studied (Grebe, 2011) (Figure 2).

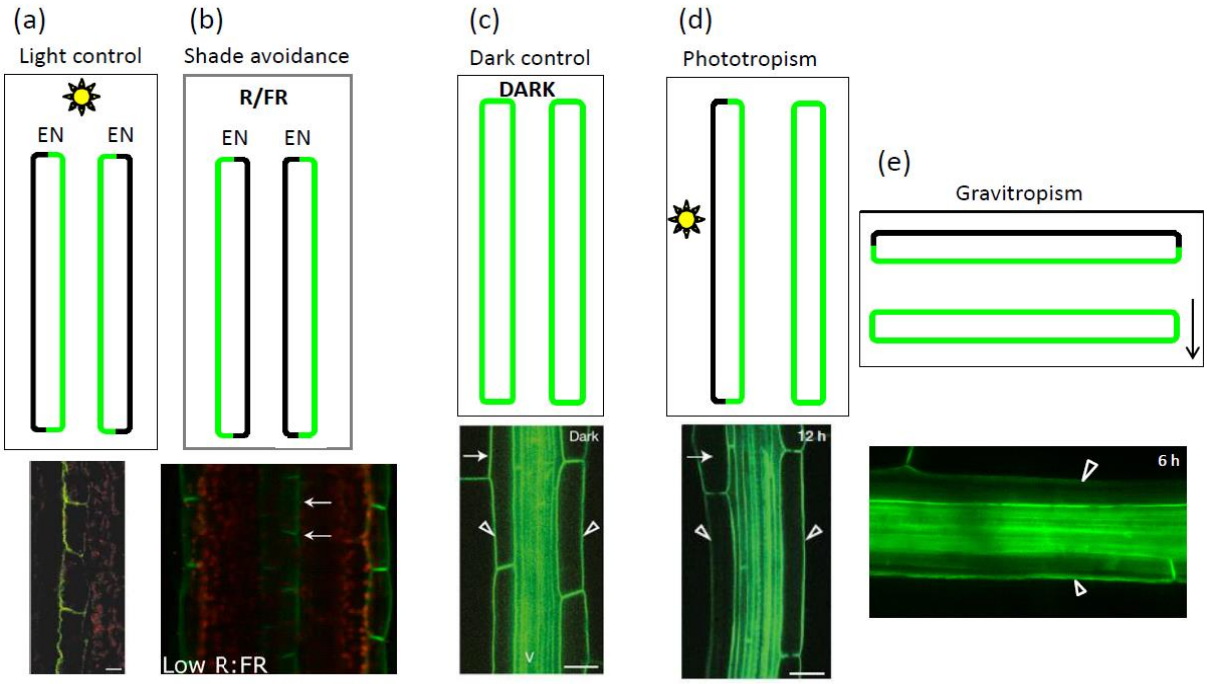

Figure 2. PIN3 localization in *Arabidopsis* hypocotyls
Light- and gravity-mediated PIN3 relocalization in the endodermis cells of upper hypocotyls. PIN3 is localized on the inner-lateral side in the light (a) and relocalizes to the outer-lateral domain during the shade avoidance response (b). Arrows point the outer lateral endodermal cell side. In the dark, PIN3 is apolarly localized (c). PIN3 gradually relocalizes to the inner-lateral cell side at the illuminated hypocotyl side after unilateral light (d) or at the upper hypocotyl side after gravity stimulation (e). Arrowheads point the outer endodermis membranes. Arrows point the light direstion (c,d) and gravity direction (e) Arrowheads point the outer endodermis membranes. Arrows point the light direction (c,d) and gravity direction (e) (*Friml et al., 2002; Keuskamp et al., 2010a; Ding et al., 2011; Rakusová et al., 2011*)

In summary, PIN proteins play an important role in the regulation of auxin fluxes during tropic responses and shade avoidance. As the main player seems to be PIN3, we will further focus on the cell polarity regulation of the PIN3 protein in hypocotyl endodermal cells.

PIN (RE)POLARIZATION DURING TROPISMS

The adaptation of growth to changing environmental conditions with complex developmental reprogramming often involves resetting of the developmental fate and polarity of cells within differentiated tissues (Kleine-Vehn and Friml, 2008). Although some components of the signalling pathways induced by light or gravity stimuli are known, the links with auxin redistribution and regulation of PIN localization are still largely missing (Ding et al., 2011; Rakusová et al., 2011). The PIN polarity establishment is regulated at several levels, notably polar secretion (Łangowski et al., unpublished), constitutive endocytic recycling (Geldner et al., 2001), and transcytosis (Kleine-Vehn et al., 2008a; 2008b). The subcellular

dynamics enable rapid changes in PIN polarity that are essential for the fast redistribution of auxin fluxes in response to stimuli, such as light or gravity.

Endocytic recycling

PM-localized PIN proteins are constitutively internalized by clathrin-mediated endocytosis (Dhonukshe et al., 2007) and recycled back to the PM (Geldner et al., 2001). In plants, clathrin-dependent membrane trafficking is critical for the polar PIN distribution and plays a role during gravitropism of roots and hypocotyls (Kitakura et al., 2011; Kleine-Vehn et al., 2011). The clathrin function is required for the rapid PIN3 internalization following a gravity stimulus in roots (Kleine-Vehn et al., 2010) and hypocotyls (Rakusová et al., manuscript in preparation) to possibly create an intracellular PIN3 pool to be redirected to the preferred cell side.

Both PIN internalization and recycling back to the PM depend on the activity of ADP-ribosylation factor (ARF) GTPases and guanine-nucleotide exchange factors (GEFs) (Geldner et al., 2003; Tanaka et al., 2009; 2013) and their counter-partner ARF GTPase-activating proteins (GAPs- (Naramoto et al., 2009). The ARF GEF GNOM is involved in PIN3 trafficking during photo- and gravitropic signalling in roots and hypocotyls (Ding et al., 2011; Kleine-Vehn et al., 2010; Rakusová et al., 2011). Surprisingly, the chemical inhibition of the GNOM function interferes with the gravity- or light-induced destabilization of PIN3 at the outer PM (Ding et al., 2011; Rakusová et al., 2011). This observation suggests that GNOM my play an additional role on the PM in hypocotyl endodermal cells, apart from its described roles in the endosomes.

Other ARF GEFs and ARF GAPs have been shown to be PIN trafficking regulators (Tanaka et al., 2009, Naramoto et al., 2009). The early endosomal component markers, the ARF GEF BFA-visualized endocytic trafficking defective1 (BEN1), BEN2 and syntaxin-binding protein 1-like 1 (SEC1-/mammalian uncoordinated-18 (MUNC18) proteins are involved in distinct steps of early endosomal trafficking. BEN1 and BEN2 are collectively required for polar PIN localization, for their dynamic repolarization, and, consequently, for auxin response gradient formation and auxin-related developmental processes (Tanaka et al., 2013). GTP exchange factors on the ADP-ribosylation factors GNOM-LIKE1 (GNL1) regulate trafficking at the Golgi apparatus and *trans*-Golgi network/early endosome (TGN/EE) (Teh and Moore, 2007). BFA-visualized exocytic trafficking defective1 (BEX1), a member of the ARF1 gene family, localizes to the TGN/EE and Golgi apparatus, acts synergistically with the ARF GEF BEN1/HOPM Interactor 7 (MIN7) and is important for PIN recycling to the PM (Tanaka et al.,

2014). BEX5 codes for Ras genes from rat brainA1b (RabA1b), a member of the large RabA GTPase class, and localizes to the TGN/EE compartment and plays a role in PIN protein trafficking, acts on trafficking processes distinct from GNL1, presumably by regulating trafficking from the TGN/EE to the PM (Feraru et al., 2012). The roles of GNL1, BEN1, BEN2, BEX1, and BEX5 in PIN1 and PIN2 trafficking have been described, but a possible role in during tropic responses and PIN3 trafficking has still to be assessed. These findings highlight the indispensable roles of EE components and endosomal trafficking in the regulation of PIN polarity and plant architecture, but their function in PIN3 trafficking has not been unravelled yet.

Role of cytoskeleton in tropic responses

The role of cytoskeleton in the distinct stages of the gravitropic response is a subject of intensive research (Blancaflor, 2002, 2013; Morita and Tasaka, 2004). Depolymerization of actin microfilaments (MFs) reduces the hypocotyl growth rates, but after a prolonged time leads to gravitropic overbending of the hypocotyls influencing gravity sensing and the cell elongation. Therefore, actin might serve as a negative regulator of gravitropism, possibly by preventing a too fast sedimentation of statoliths (Nakamura et al., 2011; Yamamoto and Kiss, 2002). On the contrary, in roots, the actin cytoskeleton is required for PIN3 recycling during gravitropism (Friml et al., 2002) and for endomembrane cycling of PIN proteins in general (Geldner et al., 2001).

Previous data indicate that the microtubule (MT) cytoskeleton is involved in the hypocotyl gravitropic response. Blue-light illumination initiates rapid MT redirection from transversal to longitudinal orientation on the lighted side, probably contributing to phototropic bending (Lindeboom et al., 2013). Longitudinally oriented cortical array will likely inhibit cell elongation through deposition of longitudinal cellulose microfibrils. As auxin accumulates on the shaded side during phototropism and lower side during gravitropism, here we would expect stimulated transversal orientation of MT to induce elongation also in the case of gravitropism. But recent data showed that auxin application causes a very rapid re-orientation of MT from transversal to longitudinal (Chen et al., 2014). On the other hand, auxin was reported to induce transversal orientation of MT in **light** grown hypocotyls (Vineyard et al., 2013), but both the phototropic and gravitropic hypocotyl experiments are performed using etiolated hypocotyls. Therefore the role of auxin in the MT orientation during tropisms remains an open question, but evidently light and auxin signalling converge on the MT cytoskeleton.

Taken together, actin and the MT cytoskeleton are important components at different stages of tropic responses. The MT cytoskeleton is generally involved in hypocotyl responses to changing light and gravity by their effect on cells elongation, whereas the actin cytoskeleton would be additionally required for PIN3 re-polarization and gravity sensing.

PIN3 transcytosis during tropisms

The vacuolar targeting of PIN2 that is enhanced in root epidermal cells during gravity responses, presumably maintains the asymmetric auxin distribution (Baster et al., 2013; Kleine-Vehn et al., 2008c), but vacuolar PIN3 targeting is not influenced during the gravity response (Kleine-Vehn et al., 2010). PIN proteins can be targeted to the vacuole to be degraded (Kleine-Vehn et al., 2008c), but the importance of gravity-induced degradation during the PIN3 relocation after gravistimulation in hypocotyls is not mandatory (Rakusová et al., 2011; Kleine-Vehn et al., 2010). Therefore it seems that, the mechanism of PIN3 re-polarization relies on relocation of the existing PIN3 pool.

Relocation-like mechanisms, generally termed transcytosis, combine endocytic recycling and recruitment into distinct polar targeting pathways (Kleine-Vehn et al., 2008a; 2008b). Light or gravity modulate multiple steps of the PIN3 trafficking, leading to PIN3 transcytosis that is physiologically important for redirection of auxin fluxes during tropic responses (Ding et al., 2011; Kleine-Vehn et al., 2010; Rakusová et al., 2011). PIN proteins have to re-localize fast in response to environmental changes. The relevant trafficking machineries must be targeted by thus far not much explored regulations based on external stimuli, such as light/shade or gravity. Phosphorylation might be one of the key components that regulate the directional trafficking of PIN proteins during the tropic responses.

Polarity regulation by phosphorylation

Reversible phosphorylation of PIN proteins by the serine/threonine protein kinase PINOID (PID) and protein phosphatase 2A (PP2A) is important for targeting PIN proteins to the proper apical or basal PMs (Benjamins et al., 2001; Friml et al., 2004; Huang et al., 2010; Michniewicz et al., 2007; Zhang et al., 2010). The AGC3 Ser/Thr protein kinase family, comprising PID, WAG1, and WAG2 is also involved in the transduction of signals dependent on phototropism and gravitropism. PID-mediated phosphorylation regulates GNOM-dependent transcytosis of PIN proteins (Ding et al., 2011; Kleine-Vehn et al., 2010; Kleine-Vehn et al., 2009), thereby affecting the PIN3 relocation in response to light (Ding et al., 2011; Zhang et al., 2010) and gravity (Rakusová et al., 2011).

The recently described D6 PROTEIN KINASE (D6K) subfamily of AGCVIII kinases directly regulates the PIN-mediated auxin transport during tropism, independently from PID (Barbosa et al., 2014, Zourelidou et al., 2014). The D6K-mediated PIN3 phosphorylation promotes auxin transport in the hypocotyls during the phototropic bending response by regulating PIN activity. D6K is also able to directly phosphorylate PIN3 *in vitro*. Interestingly, the PIN3 polarizes correctly after phototropic stimulation in the *d6pk* mutants, but the reduced PIN3 phosphorylation leads to inactivity of PIN3, which in turn results in impaired auxin redistribution and reduced phototropism (Willige et al., 2013). PIN3 auxin transport **activity** can be regulated by D6K and PID kinases (Barbosa et al., 2014; Zourelidou et al., 2014) which could represent an additional layer of auxin transport regulation during tropic responses, in adition to the control of auxin fluxes by polar localisation of auxin transporters. Obviously, both layers are needed to achieve the creation of auxin maxima required for tropic responses.

In roots, phosphorylation leads to apicalization of PIN proteins (Friml et al., 2004; Michniewicz et al., 2007), whereas in hypocotyl endodermal cells, phosphorylation seems to lead to outer-lateralization (Ding et al., 2011; Rakusová et al., 2011). Thus, the outer lateral and the inner lateral domains of hypocotyl endodermal cells would correspond to the apical and the basal domains of root cells.

CONCLUSION

The regulation of the PIN localization and subsequent auxin flow during tropisms is the subject of intensive research. Here, we summarize recent insights into the cellular mechanism of the PIN3 auxin transporter polarization in response to environmental stimulations of *Arabidopsis* hypocotyls. Somewhere in the complex mechanism of PIN3 trafficking, light sensing or statolith sedimention has to provide the directional information that indicates the shade side or the bottom of the cell. Whether this identification occurs at the level of the sorting endosomes, where PIN3 is recruited to the polar recycling pathway, at the level of vesicle fusion with the lower PM, or whether all the polar recycling trafficking routes are rearranged in response to light stimuli or statoliths sedimentation, remain a fascinating question for future research.

Although PIN3 relocates after both light or gravity stimulation, its new polarity corresponds well with the auxin flow redirection, and mutants defective in PIN3 relocation have tropism defects, the direct evidence that this process is crucial for tropisms is still missing. The

ultimate proof would require a PIN3 protein version that still transports auxin, but is not relocated after light or gravity stimulations.

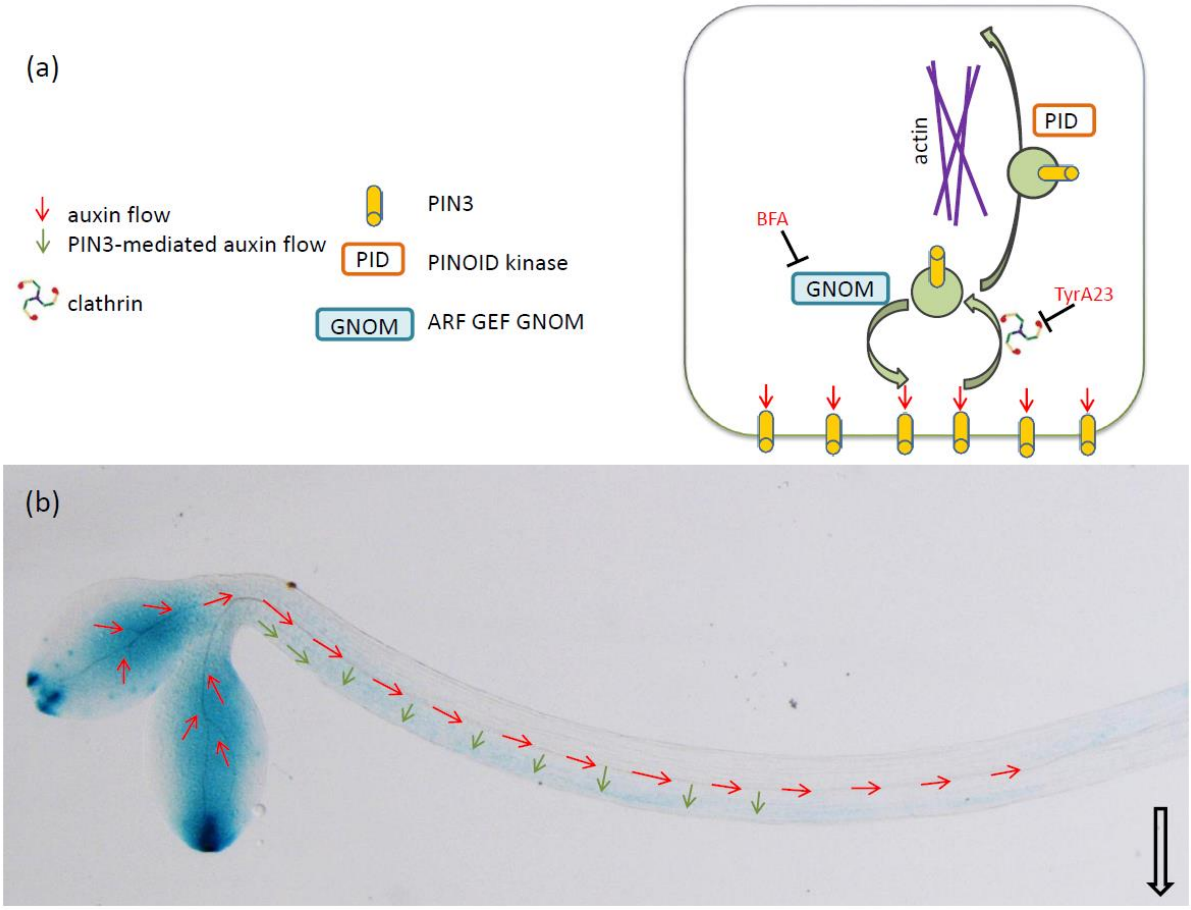

Figure 3. Intracellular PIN3 repolarization regulates auxin flow during gravitropism of hypocotyls
(a) Intracellular targeting pathways for PIN3 polar distribution during the gravitropic response. PIN3 undergoes dynamic translocation between different cell sides. Inhibition of clathrin-mediated endocytosis or the GNOM-dependent outer-PM trafficking prevents the PIN3 relocation. PINOID (PID)-dependent phosphorylation of PIN3 may affect its targeting to the inner-lateral cell side. Following gravistimulation, PIN3 relocalizes rapidly to the lower side of hypocotyl endodermis cells and, thus, redirects the auxin flow towards the lower side of the hypocotyl. (b) PIN3-mediated asymmetric auxin response during hypocotyl gravitropism as visualized by the *DR5::GUS* auxin response reporter. The arrows indicate the auxin stream direction. BFA (brefeldin A); TyrA23 (Tyrphostin A23).

Regulation of the PIN3 polarity in hypocotyl endodermal cell could be a good model system to study cell polarity regulations in general, because the PIN3 localization is inner lateral under light conditions (Friml et al., 2002), apolar in the dark (Ding et al., 2011), and outer lateral in the shade (Keuskamp et al., 2010) and switches to the inner-lateral PM side after gravity or light stimulation (Ding et al., 2011; Rakusová et al., 2011) (Figure 2.). Other important open questions concern the exact mechanism for the translation of the light and gravity perception into the directional PIN3 transcytosis. These questions should be addressed by identification of new components and mechanisms involved in tropic bending or polar sorting and trafficking.

Finally, another interesting but largely unexplored issue is the prevention of the tropic overbending. It remains entirely unclear by which mechanism the tropic response is terminated at precisely the right moment. This could bring more information about the PIN-mediated auxin accumulation and tropic bending regulation in general.

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Chapter 2.

Polarization of PIN3-dependent auxin transport for hypocotyl gravitropic response in Arabidopsis thaliana

Adapted from

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HR, GBJ and JF initiated the project and designed most of the experiments, HR carried out the experiments. GBJ performed bending kinetics analyses. MV provided line SCR::PIN3-YFP. JF, HSR, AD and, BE discussed the experimental procedures. All authors analysed and discussed the data; HR wrote the manuscript and JF assisted manuscript writing. All authors saw and commented on the manuscript.

Polarization of PIN3-dependent auxin transport for hypocotyl gravitropic response in *Arabidopsis thaliana*

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Abstract

Gravitropism aligns plant growth with gravity. It involves gravity perception and the asymmetric distribution of the phytohormone auxin. Here we provide insights into the mechanism for hypocotyl gravitropic growth. We show that the *Arabidopsis thaliana* PIN3 auxin transporter is required for the asymmetric auxin distribution for the gravitropic response. Gravistimulation polarizes PIN3 to the lower side of hypocotyl endodermal cells, which correlates with increased auxin response at the lower hypocotyl side. Both PIN3 polarization and hypocotyl bending require the activity of the trafficking regulator GNOM and the protein kinase PINOID. Our data suggest that gravity-induced PIN3 polarization diverts the auxin flow to mediate the asymmetric distribution of auxin for gravitropic shoot bending.

Introduction

Plants display remarkable abilities to adapt their physiology and development in response to environmental stimuli. Directional growth responses (tropisms) belong to the visually most obvious adaptation responses. Phototropism and gravitropism allow both shoots and roots to direct their growth in response to light and gravity, respectively (Estelle, 1996; Holland *et al.,* 2009). These adaptive growth adjustments allow optimization of organ positioning for better light perception in the case of shoots and for better water or nutrient acquisition in the case of roots. Therefore, shoots show a negative gravitropism and grow upwards, whereas roots show a positive gravitropism manifested by their downward growth. Accepted models for tropisms are based on the classical Cholodny-Went theory in which the differential distribution of the plant signalling molecule auxin underlies the unequal growth at the two sides of a bending organ (Went, 1974). Accordingly, an asymmetry in auxin distribution and auxin response has been detected in gravistimulated organs of various plant species. This asymmetric auxin distribution during tropisms is triggered by directional, intercellular auxin transport (Briggs, 1963; Young *et al.*, 1990; Epel *et al.,* 1992; Luschnig *et al.*, 1998; Rashotte *et al*., 2000; Friml *et al.*, 2002a) that is mediated by transporters of the AUX/LAX (Bennett *et al.*, 1996; Yang *et al.*, 2006), PGP (Geisler and Murphy, 2006), and PIN (Petrášek *et al.*, 2006) families (Yang and Murphy, 2009). The directionality of the polar auxin transport is determined by a polar, subcellular, localization of PIN auxin efflux carriers (Wiśniewska *et al.*, 2006). However, how the auxin transport is regulated by gravity to generate the asymmetry in the auxin distribution remains unclear.

In plants, gravity is perceived by sedimentation of specialized starch-containing organelles (statoliths) in cells of the root cap and shoot endodermis (Morita, 2010; Morita and Tasaka, 2004). In the gravity-sensing root cap cells, PIN3 is localized symmetrically but after the gravity-induced statolith sedimentation, it polarizes and accumulates predominantly at the lower side of cells (Friml *et al.*, 2002a; Harrison and Masson, 2008). This asymmetric PIN3 localization suggests that the auxin flow is redirected towards the lower side of root where auxin is known to accumulate in response to gravistimulation (Perrin *et al.,* 2005). Nonetheless, the mechanism of the PIN3 relocation and its possible requirement for asymmetric auxin distribution, remain unclear.

Here we analyze the role of PIN3-dependent auxin transport during the gravitropic response of *Arabidopsis thaliana* hypocotyls. We show that PIN3 in endodermal cells of hypocotyls is needed for the auxin redistribution and that it polarizes in response to gravity.

Furthermore, we demonstrate that the established vesicle trafficking and cell polarity regulators are necessary for both PIN3 polarization and hypocotyl gravitropism.

Results

PIN3 is required for asymmetric auxin distribution and hypocotyl gravitropism

Multiple genetic and pharmacological experiments have demonstrated that the inhibition of the auxin transport interferes with tropisms (Perrin *et al.,* 2005). Specifically, *pin3* mutants had reduced hypocotyl gravitropic responses (Friml *et al.,* 2002a). First, we tested the contribution of PIN3 to the hypocotyl gravitropic response by detailed kinematic analyses of the hypocotyl bending. As expected, the bending in *pin3* loss-of-function seedlings was much less pronounced than that in the wild type (Figure 1a,b,d). In contrast, mutants of the closest homologues *pin4*, and *pin7* and the *pin4 pin7* combination did not show any defects in the hypocotyl gravitropism (Figure 1c,e). Nonetheless, multiple mutant combinations of *pin3* with *pin4* and *pin7* including *pin3 pin4 pin7* did show increasingly stronger defects in hypocotyl gravitropism (Figure 1e) suggesting redundant action of different PIN auxin transporters in hypocotyl gravitropism. Thus, unlike in root gravitropism (Kleine-Vehn *et al.,* 2010), PIN3 seems to be the major factor in the hypocotyl because the closest homologues PIN4 and PIN7 do not seem to play an important role in the hypocotyl gravitropism despite they redundantly contribute to some extent in absence of the PIN3 function. The strong developmental defects and even lethality of higher order *pin* mutants (Friml *et al.,* 2003; Blilou *et al.,* 2005) do not allow to test if PIN proteins are solely auxin transporters required for hypocotyl gravitropism, however, strong effects of the auxin transport inhibitors (Jensen *et al.,* 1998; Friml *et al.,* 2002a) demonstrate an essential role of auxin transport. Additionally, the auxin transporters from the PGP family that functionally co-operates with the PIN-dependent transport (Blakeslee *et al.,* 2007; Mravec *et al.,* 2008) contribute to the gravitropic response (Rojas-Pierce *et al.,* 2007). Overall, these results revealed that PIN3 is the major contributor for PIN-dependent auxin transport during the hypocotyl gravitropic response with redundant contribution of other related PIN transporters.

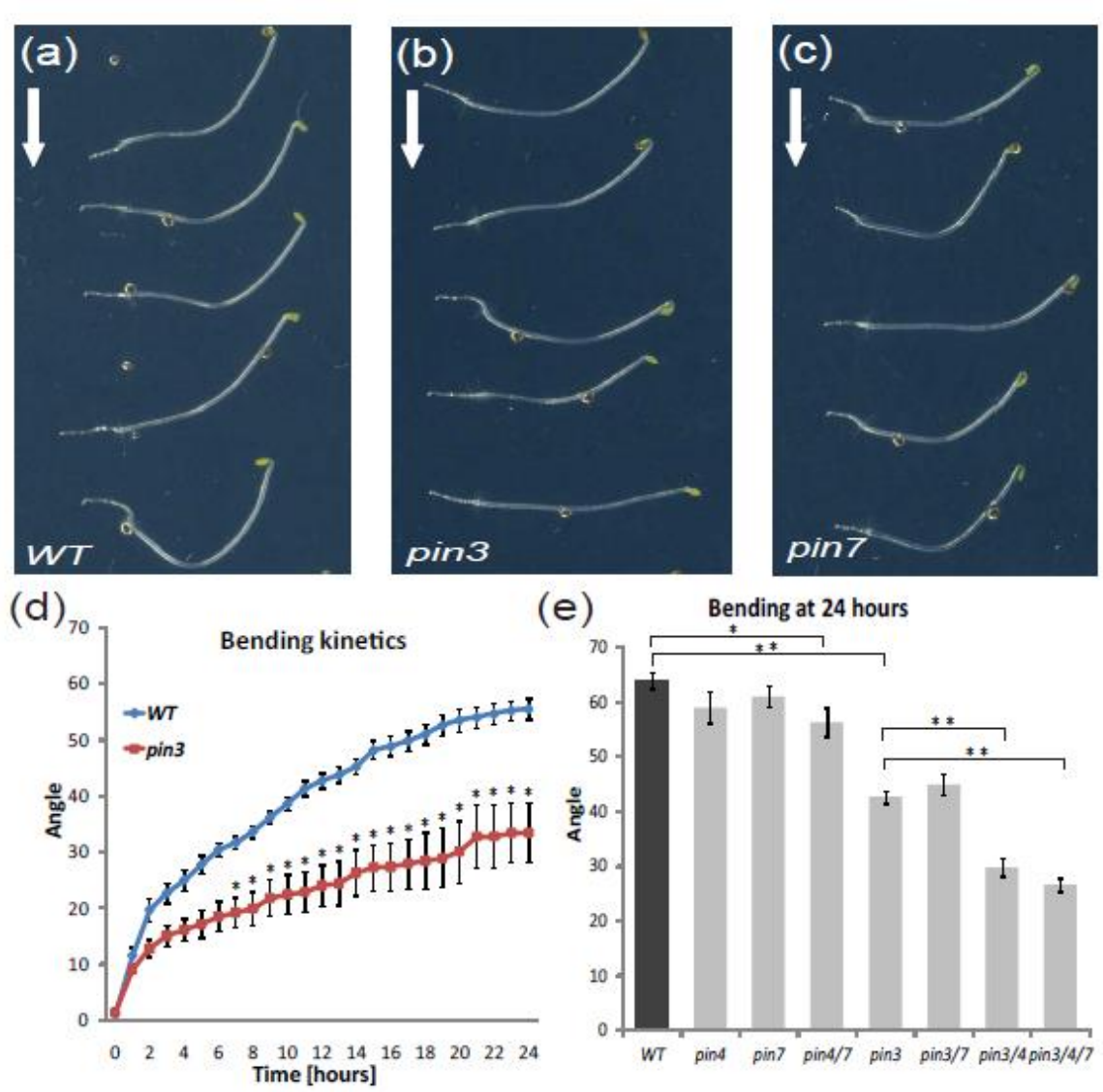

Figure. 1. Hypocotyl gravitropic response of *pin* mutants.
(a-c) Bending of wild-type (a), *pin3* (b) and *pin7* (c) hypocotyls after 24 h of gravistimulation. Arrows mark gravity direction.
(d) Hypocotyl bending kinetics of the wild type and *pin3* mutants. Hypocotyl curvatures were measured every hour and average curvatures were calculated. Values are the average of three biological replicates (n>10 per time point on each replicate). Error bars are SE (Student's *t* test, *P<0,05).
(e) Hypocotyl bending of the wild type, *pin4*, *pin7*, *pin4 pin7*, *pin3*, *pin3 pin7*, *pin3 pin4* and *pin3 pin4 pin7* mutants. Hypocotyl curvatures were measured after 24 h and average curvatures were calculated. Values are the average of three biological replicates (n>10 per time point on each replicate). Error bars are SE (Student's *t* test between wild-type and *pin4 pin7*, **P*<0,02; Student's *t* test between wild-type and *pin3*, *pin3 pin7*, *pin3 pin4* and *pin3 pin4 pin7* mutants, ***P*<0,0001).

Next, we investigated whether the PIN3 function is required for generating the asymmetric auxin distribution following gravistimulation. We used the auxin-responsive *DR5rev::GFP* line, of which the spatial activity pattern has been shown to correlate with auxin distribution as inferred from IAA immunedetection in embryos and lateral organs (Benková *et al.*, 2003; Friml *et al.*, 2003) and in gravistimulated roots (Ottenschläger *et al.*, 2003).

Consistent with previous reports (Friml *et al.*, 2002a), we detected an asymmetric DR5 activity across the gravistimulated hypocotyls with higher relative fluorescence intensity at the lower side (Figure 2a,c,d). Notably, when the PIN3 activity was compromised in *pin3* mutants, the asymmetry in *DR5rev::GFP* activity was significantly less pronounced in gravistimulated hypocotyls as compared with the wild type (Figure 2b,e,f,g). These observations confirm a role for PIN3 in hypocotyl gravitropism and show that PIN3 activity is required for the generation of the lateral auxin gradient underlying the hypocotyl gravitropic response.

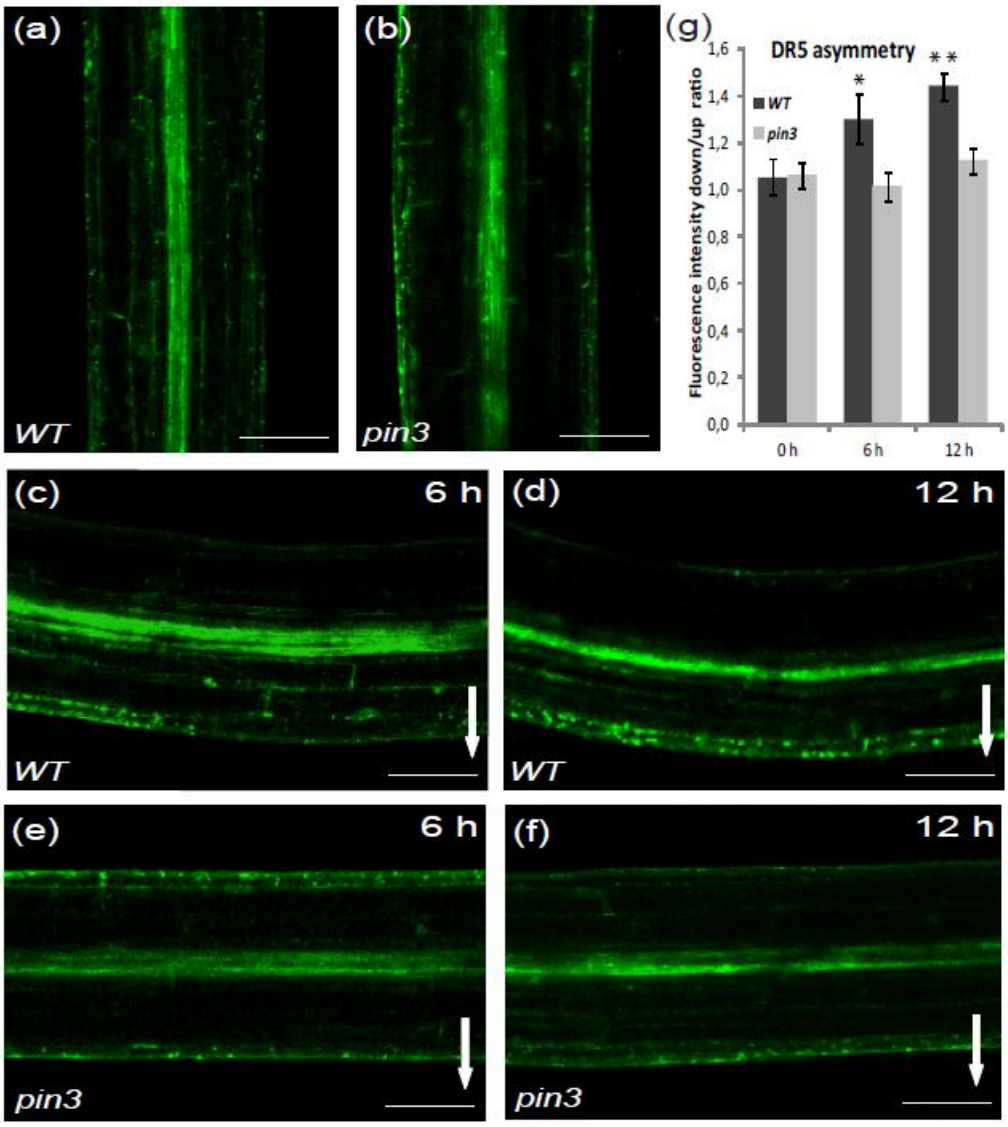

Figure 2. **Asymmetric distribution of the auxin responses during hypocotyl gravitropism.**
(a-f) Expression of *DR5rev::GFP* auxin response reporter in wild-type (a, c, d) and *pin3* (b, e, f) hypocotyls. Controls without stimulation (a, b), and stimulation for 6 h (c, e) or 12 h (d, f). More pronounced asymmetry in the DR5 activity between upper and lower side of wild type hypocotyls after gravistimulation (c, d) as compared to *pin3* mutant (e, f). Arrows mark direction of gravity. Scale bar: 100 μm.
(g) Quantification of *DR5rev::GFP* fluorescence intensity at the lower side of hypocotyl in the wild type and *pin3* mutant after different times of gravistimulation. Values are the average of three biological replicates (n>10 per time point on each replicate). Error bars are SE (Student's *t* test **P*<0,1, ***P*<0,001).

Gravity induces rearrangements of PIN3 localization in hypocotyl endodermis cells

Next, we studied the mechanism by which gravity triggers the redirection of the PIN3-dependent auxin flow to the lower side of hypocotyls. Gravistimulation has been shown to induce changes in the polar PIN3 localization in roots where PIN3 relocates towards the bottom side of columella cells following gravistimulation (Friml *et al.,* 2002a; Harrison and Masson, 2008; Kleine-Vehn *et al.,* 2010). Therefore, we analyzed the effect of gravistimulation on the localization of the functional PIN3-GFP (Žádníková *et al.,* 2010) in hypocotyls. As PIN3 is strongly expressed in the hypocotyl endodermis (starch sheath) (Friml *et al.,* 2002a), a strong PIN3-GFP signal was detected at both the inner and outer lateral sides of endodermal cells and weaker expression was found in cortical and epidermal cells (Ding *et al*., 2011; Figure 3a). We performed the experiments in dark to avoid any influence of light on PIN3 localization (Ding *et al*., 2011). After gravistimulation by placing the hypocotyl horizontally, an asymmetry in the PIN3 localization gradually developed in endodermal cells: A strong PIN3-GFP signal persisted at outer sides of endodermal cells at the lower hypocotyl side, whereas at the outer cell sides at the upper hypocotyl side it gradually became weaker (Figure 3a-c,g-j). This generation of asymmetry in PIN3 localization following gravistimulation hereafter termed "gravity-induced PIN3 polarization" could be visualized mainly in the outer sides of endodermal cells because inner sides of endodermal cells were largely obscured by the strong GFP signal in the middle vascular cylinder. Therefore, measurements were done comparing the PIN3 signal in the outer sides of endodermal cells at the upper versus lower side of endodermal cells. Notably, these changes of PIN3 localization following gravistimulation were not observed along the whole hypocotyl but were restricted to the gravity-responding, more apical part (Figure 3k). Genetic data pointed to PIN4 as an additional regulator of the gravitropic response in the absence of PIN3. However, PIN4 expression level in the hypocotyls was very weak and only present in epidermal cells (data not shown). The other closer homolog of PIN3, PIN7, which does not play a major role in hypocotyl gravitropism (Figure 1c,e) was present in endodermal cells. However, in contrast to PIN3-GFP, PIN7-GFP did not show any visible changes in polarity (Figure 3d-f).

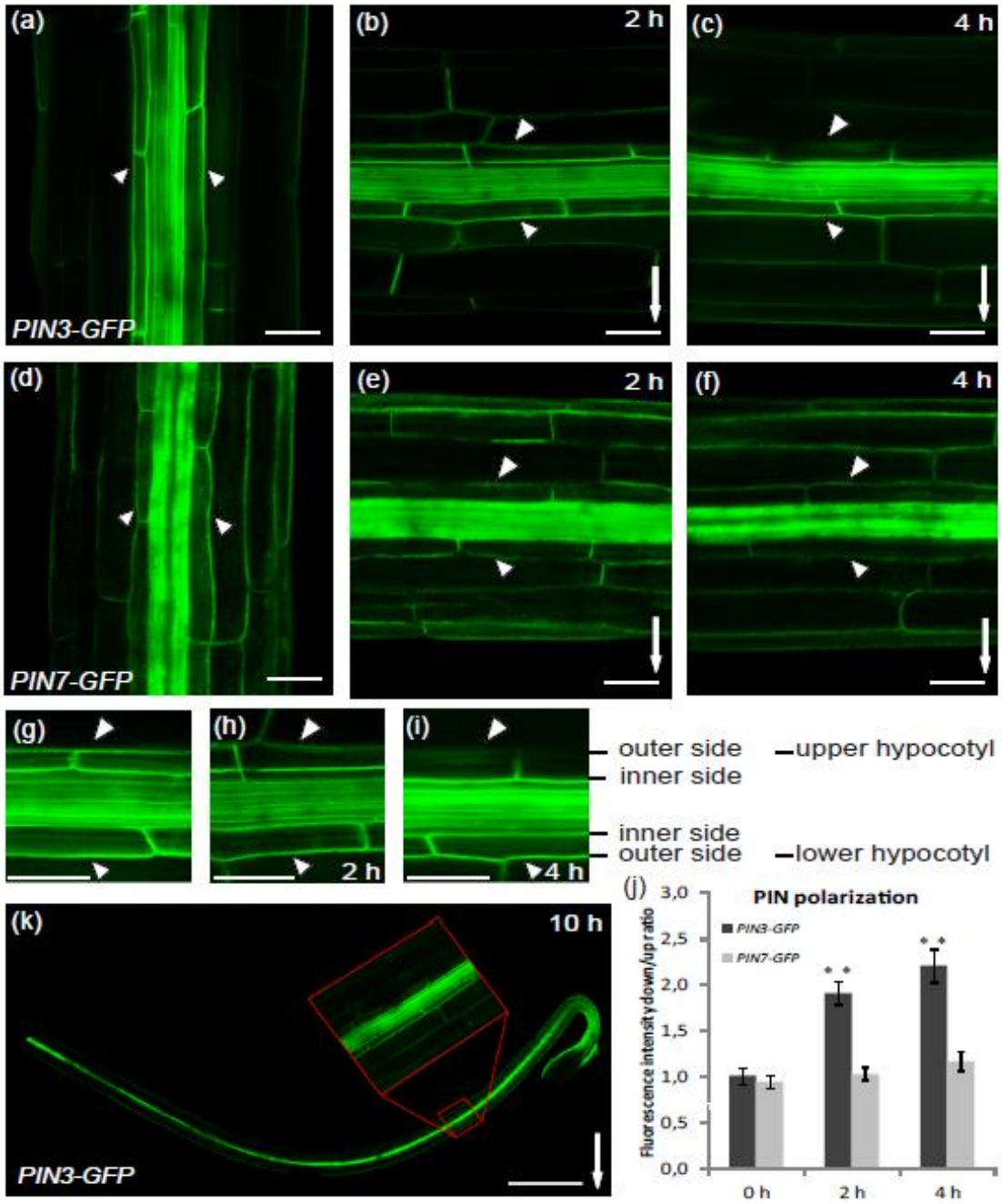

Figure 3. **Gravity-mediated PIN3 polarization.**
(a-c) PIN3-GFP in the upper, gravity-responsive part of hypocotyl. The GFP signal was detected at the outer and inner sides of endodermal cells (a). Following gravistimulation, the PIN3-GFP localization disappeared from the upper side of endodermal cells of the upper hypocotyl half but was difficult to visualize in the lower hypocotyl half due to the strong GFP fluorescence in inner vascular cylinder cells (b, c).
(d-f) PIN7-GFP in the upper, responsive hypocotyl part. The GFP signal was visible at the outer and inner membranes of endodermal cells (d). Following gravistimulation, no pronounced change in the PIN7-GFP localization was seen (e, f). Scale bar: 50 μm.
(g-i) Details of gravity-induced PIN3-GFP polarization. Gradual disappearance of the GFP signal at the outer sides of endodermis cells in the upper hypocotyls half (h, i). Non-stimulated control (g). Scale bar: 50 μm.
(j) Ratio of PIN3-GFP and PIN7-GFP fluorescence intensities at the outer side of endodermis cells at the lower versus upper hypocotyl side after different times of gravistimulation. Values are the average of three biological replicates (n>10 per time point on each replicate). Error bars are SE. (Student's *t* test, ***P*<0,0001). (k) Approximate position at the hypocotyl where gravity-induced PIN3-GFP polarization was observed. Arrows and arrowheads indicate gravity direction and the GFP localization at the outer sides of endodermal cells, respectively. Scale bar: 0,8 mm.

To test specifically the role of endodermal PIN3 in hypocotyl gravitropism and also better visualize PIN3 polarity changes also at the inner sides of endodermis cells, we placed the PIN3 coding sequence under the control of the *SCARECROW* (*SCR*) promoter that drives expression exclusively in endodermis (Wysocka-Diller *et al.*, 2000). The expression level of *SCR::PIN3-YFP* in the endodermis of *pin3* mutant was visibly lower but showed normal, apolar signal similar to that observed in *PIN3::PIN3-GFP* hypocotyls (Figure 4a). Following gravistimulation, we measured the PIN3-YFP fluorescence intensity and observed progress of the asymmetry in PIN3-YFP localization reflected by gradual disappearance of the signal from the upper sides of endodermal cells (outer side in the upper half and inner side at the lower half). Importantly, the absence of the strong fluorescence signal in the vascular cylinder of *SCR::PIN3-YFP*, allowed us to observe the gravity-induced changes in PIN3 localization also at the inner sides of endodermal cells (Figure 4b,c). Notably, the *SCR::PIN3-YFP* rescued to a significant extent the gravitropic defect of *pin3* mutant hypocotyls (Figure 4d-h) strongly suggesting that the PIN3 action specifically in endodermis cells is sufficient to mediate hypocotyl gravitropic response. The incomplete rescue might be due to much weaker expression of *SCR::PIN3-YFP* as compared to *PIN3::PIN3-GFP*.

Overall, the gravity-induced rearrangements in the PIN3 localizations resulted in the polarization of PIN3 towards the lower side of endodermal cells of hypocotyls perpendicular to the gravity vector. Here, PIN3 is perfectly positioned to redirect auxin flow from the basipetal stream in the centre of the hypocotyl towards its lower side where auxin accumulates to drive growth for gravitropic bending.

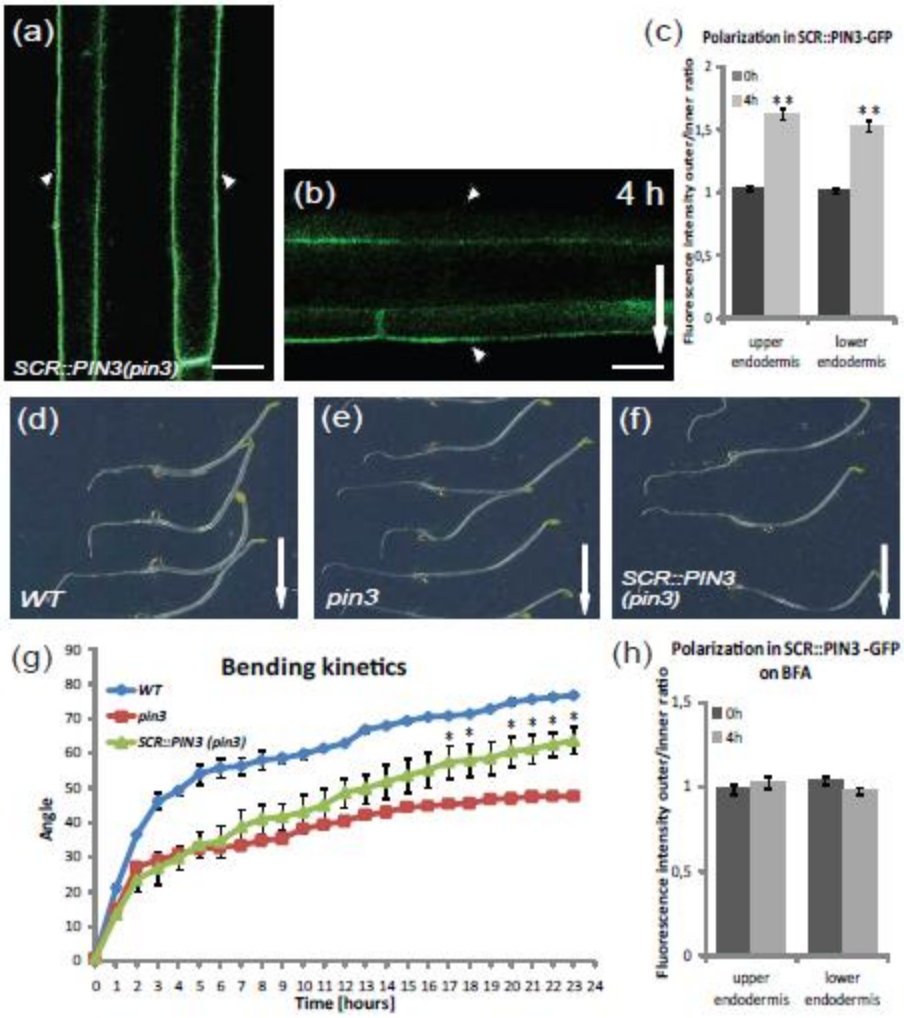

Figure 4. **Gravity-induced polarization in endodermis of *SCR::PIN3-YFP*.**
(a, b) *pin3* mutant expressing *PIN3-YFP* under the control of *SCR* promoter. PIN3-YFP localizes exclusively in the endodermal cells of the seedling. Image of the gravity-responsive hypocotyl part of *pin3* mutant carrying *SCR::PIN3-YFP* showing symmetric signal between both sides (a). Following gravistimulation, PIN3-YFP disappeared from the upper sides of endodermal cells in both upper and lower hypocotyl halves (b) Arrowheads indicate the YFP localization at the outer side of endodermal cells. Scale bar: 20 μm.
(c) Ratio of PIN3-GFP fluorescence intensities at the outer and inner sides of upper and lower endodermis cells sides after different times of gravistimulation. Values are the average of three biological replicates with at least 10 seedlings measured for each time point. Error bars are SE (Student's *t* test: ***P*<0,0001).
(d-g) *SCR::PIN3-YFP* significantly rescued the hypocotyls gravitropism defect of *pin3* after 24 h of gravistimulation (d-f).Arrows indicate gravity direction. Bending kinetics of wild-type, *pin3* and *SCR::PIN3-YFP* in *pin3* background (g). Values are the average of one biological experiment (n>10 per time point on each replicate). Error bars are SD (Student's t test: *P<0,03). Similar results where obtained with other *SCR::PIN3-YFP* lines.(H) Ratio of PIN3-GFP fluorescence intensities at the outer and inner sides of upper and lower endodermis cells side after different times of gravistimulation on BFA. Values are the average of three biological replicates (n>10 per time point on each replicate). (Student's t test show no significant differences).

Gravity induces translocation of PIN3 in hypocotyl endodermal cells

Next, we addressed the cellular mechanism underlying the PIN3 polarization in response to gravity. Conceptually, the rearrangements of the PIN3 localization can be result from *de novo* protein synthesis, degradation and translocation or combination of these processes.

First, we examined whether protein synthesis is involved in PIN3 polarization during the hypocotyl gravitropic response. We blocked protein biosynthesis with cycloheximide (CHX) and observed normal PIN3-GFP polarization following gravistimulation (Figure S1f). For longer CHX treatments, as expected, no hypocotyl growth and bending occurred (Figure S2d and S1c,g). As next we tested effect of proteolytic protein degradation using MG132 treatment that also affects differential degradation of PIN2 during root gravitropism (Abas *et al.*, 2006). The MG132 had no visible effect on gravity-induced PIN3 polarization (Figure S1d) or on hypocotyls growth or gravitropic bending (Figure S2c and S1b,e). Accordingly, the *snx1* mutant defective in PIN2 degradation (Jaillais *et al.*, 2006; Kleine-Vehn *et al.*, 2008) showed normal hypocotyl gravitropism (Figure S1h,i). These results show that *de novo* protein synthesis or proteolytic protein degradation are not the major mechanisms underlying the gravity-induced PIN3 polarisation. This suggests that similar to gravity-induced PIN3 polarization in the root cap (Kleine-Vehn *et al*., 2010), also the PIN3 polarization in hypocotyl is achieved by a translocation of PIN3 protein from the pre-existing protein pool towards the lower side of endodermal cells.

GNOM ARF GEF is required for gravity-induced PIN3 translocation

PIN proteins recycle constitutively between the plasma membrane and the endosomes (Geldner *et al.*, 2001; Dhonukshe *et al.*, 2007). An endosomal membrane-associated guanosine-nucleotide exchange factor on the ADP-ribosylation factors (ARF-GEF), GNOM, mediates PIN trafficking to the plasma membrane and is inhibited by the fungal toxin brefeldin A (BFA) (Geldner *et al.*, 2003). BFA treatment interfered with the PIN3-GFP polarization in response to gravistimulation as manifested by the persistent PIN3::PIN3-GFP and SCR::PIN3-YFP localizations at both outer and inner sides of endodermal cells (Figure 5a-c; compare to Figure 3 and 4h). Accordingly, BFA also affected the establishment of asymmetry in DR5-monitored auxin responses and hypocotyl bending after gravitropic stimulation (Figure 5g-i). These observations revealed that both PIN3 polarization in response to gravity as well as the downstream events, require action of BFA-sensitive ARF GEFs.

To test whether GNOM is the specific ARF GEF component that is needed for the BFA-sensitive PIN3 polarization, we used the *GNOM*^{M696L} line carrying the engineered BFA-resistant version of GNOM (Geldner *et al.*, 2003). In *GNOM*^{M696L} hypocotyls, the gravity-induced polarization of PIN3 (Figure 5b,c) and the gravitropic bending occurred largely normally even after the BFA treatment (Figure 5e,f), demonstrating that the inhibition of GNOM specifically causes the strong effect of BFA on the PIN3 polarization and on the

gravitropic response. As a control, we confirmed that BFA did not cause a defect in hypocotyl growth in this line (Figure S2b). Additionally, the role of GNOM was confirmed using the weak *gnom*^{R5} loss-of-function allele (Geldner *et al*., 2004) that shows strongly reduced root (Kleine-Vehn *et al*., 2010) and hypocotyl (Figure 5j,k) gravity responses.

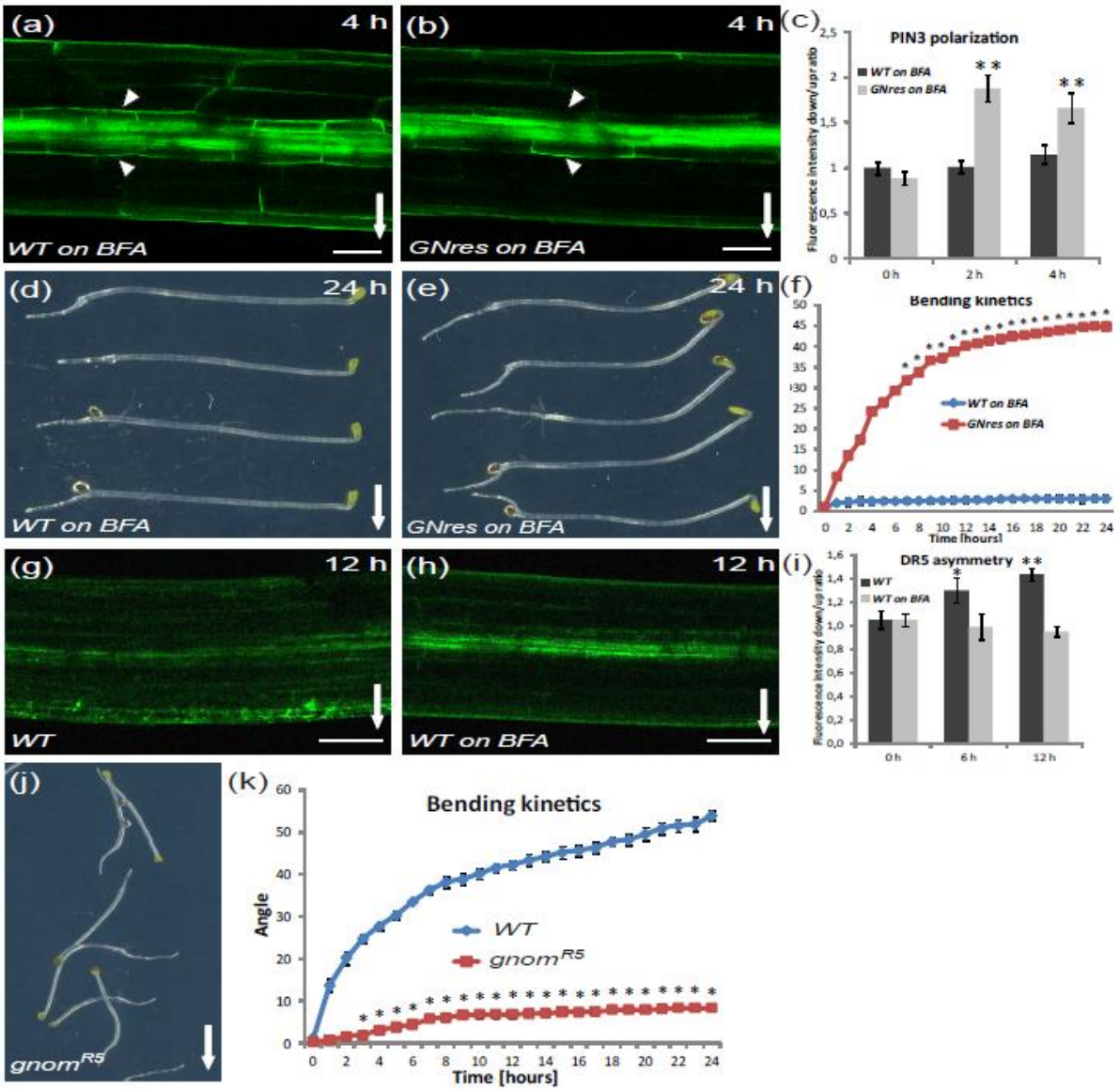

Figure 5. **ARF GEF GNOM action in gravity-induced PIN3 polarization.**
(a-c) BFA effects on PIN3-GFP polarization in hypocotyl endodermal cells after 4 h of gravistimulation. BFA treatment (50 µM) interfered with the PIN3 polarization in the wild type (a), but not in the BFA-resistant *GNOM*^{M696L} line (b). Scale bar: 100 µm. Ratio of PIN3-GFP fluorescence intensity at the outer side of endodermis cells at the lower versus upper hypocotyl side after different times of gravistimulation on BFA (c). Values are the average of three biological replicates (n>10 per time point on each replicate). Error bars are SE (Student's *t* test, ***P*<0,0001).
(d-f) BFA treatment interferes with hypocotyls bending of wild type (d), but not of the BFA-resistant *GNOM*^{M696L} line (e) after 24 h of gravistimulation. Bending kinetics of wild-type and BFA-resistant *GNOM*^{M696L} hypocotyls on BFA (f). Values are the average of three biological replicates (n>10 per time point on each replicate). Error bars are SE (Student's t test: P<0,03 (1 h to 5 h), *P<0,0001 (6 h to 24 h)).
(g-i) BFA treatment interferes with the establishment of the asymmetric *DR5rev::GFP* activity after 12 h of gravistimulation (h) as compared with the untreated control (g). Scale bar: 50 µm. Ratio of *DR5rev::GFP* fluorescence intensity at lower and upper hypocotyl sides after different times of gravistimulation (i). Values are

the average of three biological replicates with at least 10 seedlings measured for each time point. Error bars are SE (Student's *t* test, **P*<0,1, ***P*<0,0004). Arrows and arrowheads indicate gravity direction and the GFP localization at the outer sides of endodermal cells, respectively.
(j, k) Bending of wild-type and *gnom*^{R5} hypocotyls. (k) Hypocotyl bending kinetics of the wild type and *gnom*^{R5} mutant. Hypocotyl curvatures were measured every hour and average curvatures were calculated. Values are the average of two biological replicates (n>10 per time point on each replicate). Error bars are SE (Student's *t* test, *P<0,001).

Thus, our observations imply that the gravity-dependent PIN3 polarization, the downstream asymmetric auxin distribution and gravitropic response both in root (Kleine-Vehn *et al*., 2010) and hypocotyls require BFA-sensitive, GNOM-dependent trafficking. Notably, these observations further correlate the PIN3 polarization with functional gravitropic responses.

PID kinase is involved in PIN3 polarization and hypocotyl gravitropism

An important component of the PIN polarity regulation is the serine/threonine protein kinase PINOID (PID) (Christensen *et al.,* 2000; Benjamins *et al.,* 2001; Friml *et al.,* 2004; Sukumar *et al*., 2009; Ding *et al*., 2011). PID phosphorylates PIN proteins (Michniewicz *et al.,* 2007) and PIN phosphorylation is sufficient to change the polarity of the PIN localization (Zhang *et al.,* 2010; Huang *et al.,* 2010). Thus, the levels of PID-dependent phosporylation within cells largely contribute to the decision on the polar targeting of PIN proteins (Friml *et al.,* 2004; Michniewicz *et al.,* 2007). To test whether the PID-based mechanism is involved in the PIN3 polarization during gravitropic responses, we analyzed the *35S::PID* seedlings that strongly and constitutively express *PID* (Benjamins *et al.,* 2001). Gravity-dependent PIN3 polarization did not occur in the *35S::PID* plants (Figure 6a-d). Accordingly, a time-lapse analysis revealed that *35S::PID* hypocotyls have a strongly disrupted gravitropic response (Figure 6e,f) but normal hypocotyl growth (Figure S2a).

Next, we studied the hypocotyl gravitropism of *pid* loss-of-function seedlings. We used *wag1 wag2 pid* triple-mutant lines lacking activity of PID and the closest homologues WAG1 and WAG2 (Cheng *et al.,* 2008; Dhonukshe *et al.,* 2010). In contrast to gain-of-function allele, the *wag1 wag2 pid* mutant hypocotyls showed gravity-induced PIN3 polarization (Figure S3d-f) and hypergravitropic hypocotyl growth (Figure S3a-c). These observations suggest that the gravity-dependent PIN3 polarization and downstream gravitropic response involve the PID-dependent regulation of the PIN polar targeting.

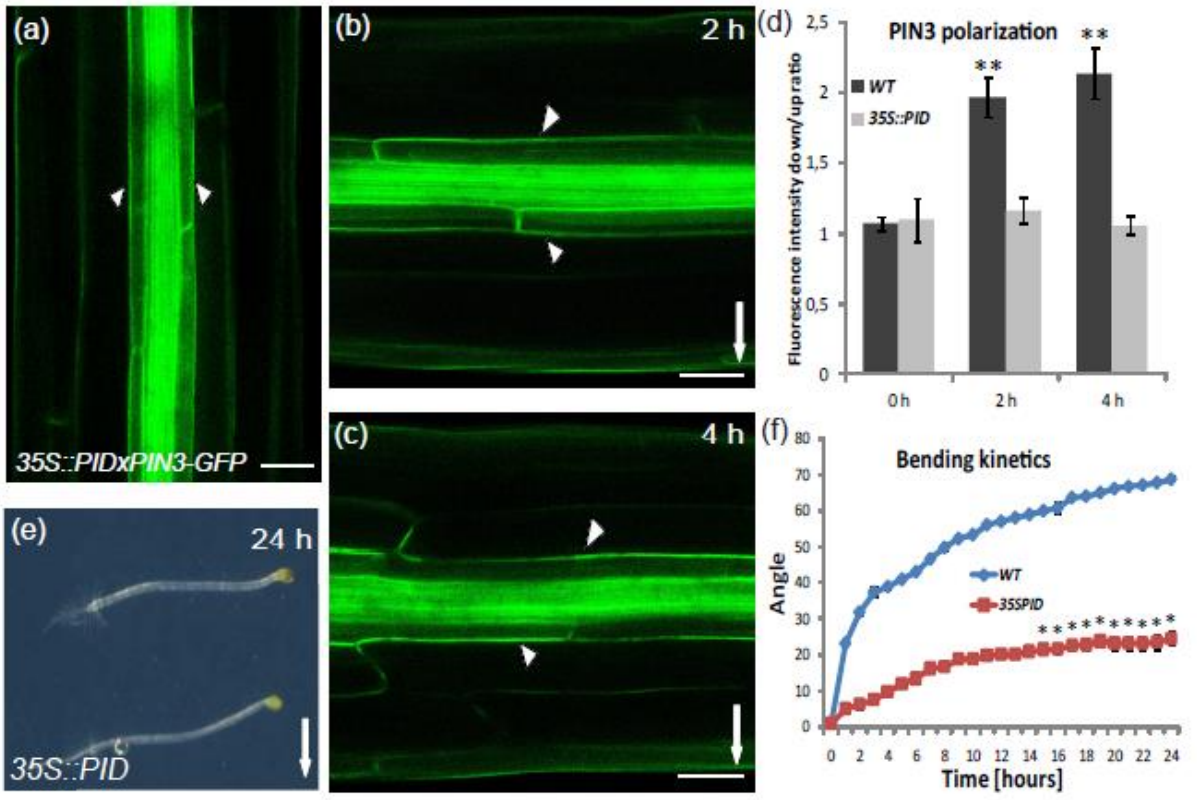

Figure 6. **PINOID action in gravity-induced PIN3 polarization.**
(a) Non-stimulated *35S::PID* control.
(b, c) No gravity-induced PIN3-GFP polarization in the *35S::PID* hypocotyls after 2 h and 4 h of gravistimulation, respectively. Arrowheads indicate the GFP localization at the outer sides of endodermal cells. Arrow indicate gravity direction. Scale bar: 50 μm.
(d). Ratio of PIN3-GFP fluorescence intensity at the outer side of endodermis cells at the lower versus upper hypocotyl side after different times of gravistimulation. Values are the average of three biological replicates with at least 10 seedlings measured for each time point. Error bars are SE (Student's *t* test: ***P*<0,0001).
(e) Reduced hypocotyl gravitropic response in the *35S::PID* line.
(f) Hypocotyl bending kinetics of the wild type and the *35S::PID* line following gravistimulation. Values are the average of three biological replicates (n>10 per time point on each replicate). Error bars are SE (Student's t test P<0,001 (1 h to 14 h), *P<0,0001 (16 h to 24 h)).

DISCUSSION

Reorientation of plant growth according to gravity anchors plants in the soil, helps roots to exploit nutrient-containing soils and orients shoots to maximize the sun exposure. The underlying mechanism involves a cascade of signalling events starting with the gravity perception and including the asymmetric distribution of the signalling molecule auxin that mediates differential growth rates, ultimately leading to the bending of the stimulated organ. However, the connections between these events are still not well understood (Perrin *et al.*, 2005; Esmon *et al.*, 2006; Muday and Rahman, 2007).

Here we provide insight into the mechanism underlying the hypocotyl gravitropism, in particular, on how the gravity signal is translated into the asymmetrical auxin accumulation,

thus aligning the directions of the gravity vector and shoot growth. In hypocotyls, gravity is perceived by the sedimentation of starch-containing amyloplasts in endodermal cells (Morita, 2010). In the same cells, the originally symmetrically localized PIN3 auxin transporter polarizes to the lower side of the endodermis in response to the gravistimulation. Such localization presumably diverts the auxin flow to the lower side of hypocotyls, where auxin accumulates and stimulates growth to promote the gravitropic bending. This PIN3 polarization in the hypocotyl requires a known component of the PIN trafficking, GNOM ARF GEF, and involve the regulator of the PIN polarity, the protein kinase PID.

This process is largely analogous to the PIN3 polarization in gravity-sensing root cap cells (Friml *et al.,* 2002a; Harrison and Masson, 2008), but root gravitropic response seems to be mediated by the redundant action of several PIN proteins (Kleine-Vehn *et al.,* 2010), whereas PIN3 seems to be the major factor in the hypocotyls. Thus, the gravity-induced polarization of PIN proteins is the common signal transduction mechanism for both positive gravitropic growth of roots and negative gravitropism of shoots. In the hypocotyls, PIN3 is mainly expressed in the endodermis and respond to gravity while its closer homologues PIN4 and PIN7 are not highly expressed there or don´t respond to gravity respectively. Hence, in a wild type situation, PIN3 is the major factor but in its absence, PIN4 can take part of the function. Notably, both PIN4 and PIN3 are also expressed in the epidermis, where they might also contribute to the gravitropic response but these epidermal roles remain still unclear.

It has been recently shown that PIN3 polarization is also involved in hypocotyl phototropic response. The light-induced PIN3 polarization in the endodermal cells appears to be at heart of this process and also depends on PIN trafficking regulators, GNOM and PID (Ding *et al.*, 2011). However, while overexpression of PID affects negatively PIN3 relocation in both, the photo- and gravi-response, the opposite effects of the *pid* triple-mutant between the two different tropic responses points out to a more complex role of PID and related kinases in gravitropism. Whereas, in phototropic response, light-mediated transcriptional repression of PID links light perception and PIN3 polarization (Ding *et al*., 2011), in gravitropism, PID activity seems to be required to fine-tune the extent of hypocotyl bending and prevent hyper-response to gravity.

The major open questions for future investigations concerns the mechanism by which the initial event in gravity perception, the statolith sedimentation, is translated into the PIN3 polarization and how PINOID-mediated phosphorylation and ARF GEF-mediated trafficking are exactly involved in this mechanism.

EXPERIMENTAL PROCEDURES

Plant material

The published transgenic and mutant lines were: *DR5rev::GFP* (Friml *et al.,* 2003); *PIN3::PIN3-GFP* (Žádníková *et al.,* 2010); *PIN7::PIN7-GFP* (Blilou *et al.,* 2005); *GNOM*^{M696L} (Geldner *et al.,* 2003); *gnom*^{R5} (Geldner *et al.,* 2004); *pin3-4* (SALK_005544); *pin4-3* (Friml *et al.,* 2002b); *pin7-1* (Friml *et al.,* 2003); and *35S::PID* (Benjamins *et al.,* 2001); *wag1 wag2 pid* (Dhonukshe *et al.,* 2010); *snx1* (Jaillais *et al.,* 2006). The *SCR::PIN3-YFP* construct was cloned using the Gateway technology. The *PIN3-YFP* genomic coding sequence (Žádníková *et al*., 2010) and the *SCR* 2500 bp promoter region were cloned into donor vectors *pDONR221* and *pDONRP4P1R,* respectively. The expression clone was generated by recombining both fragments into the expression vector *pB7m24GW* and transformed to *Arabidopsis* (Col-0). Mutant combinations with *DR5rev::GFP* and *PIN3::PIN3-GFP* were generated through genetic crosses.

Growth conditions

Seeds were sown on plates with Arabidopsis medium and stratified at 4°C for 4 days. The germination was induced by placing the plates in the light for 5-6 h that were then transferred to darkness and kept at 19°C for 4 days. For gravitropic stimulations, plates with 4-day-old seedlings were turned 90°. For confocal microscopy, a LSM 510 or Exciter confocal scanning microscope (Zeiss; http://www.zeiss.com) was used. To monitor the gravitropic response, plates were scanned 24 h after gravistimulation. Images were processed in Adobe Photoshop CS. Each experiment was done at least three times.

Real-time analysis of hypocotyl gravitropism

Bending of seedlings after gravitropic stimulation was recorded at 1-hour intervals for 1 day at 19°C. The experimental setup consisted in an infrared light source (880 nm LED; Velleman; http://www.velleman.eu) and a spectrum-enhanced camera (EOS035 Rebel Xti, 400DH) (http://www.canon.com) with built-in clear wideband-multicoated filter and standard accessories (Canon) and operated by the EOS utility software. Angles and fluorescence intensity were measured by ImageJ (NIH; http://rsb.info.nih.gov/ij). Rate of the fluorescence intensity of PIN3-GFP was comparison between the external plasma membrane sides of endodermal cells. Rate of the fluorescence of *DR5rev::GFP* was comparison between the lower

and upper side of the hypocotyl in the responsive part. Three replicates of at least 15 seedlings with synchronized germination start were processed.

Pharmacological treatments

Wild-type seedlings were germinated and grown on mock medium. BFA, MG132 and CHX treatments in the dark were done by transfer and incubation of 4-day-old etiolated seedlings on solid medium supplemented with BFA (50 µM), MG132 (25 µM) or CHX (50 µM). One hour treated seedlings on plates containing BFA, MG132 or CHX were subsequently gravistimulated for bending angle measurements or for imaging. For all comparisons, at least three independent experiments were done with the same significant results.

SUPPLEMENTAL INFORMATION

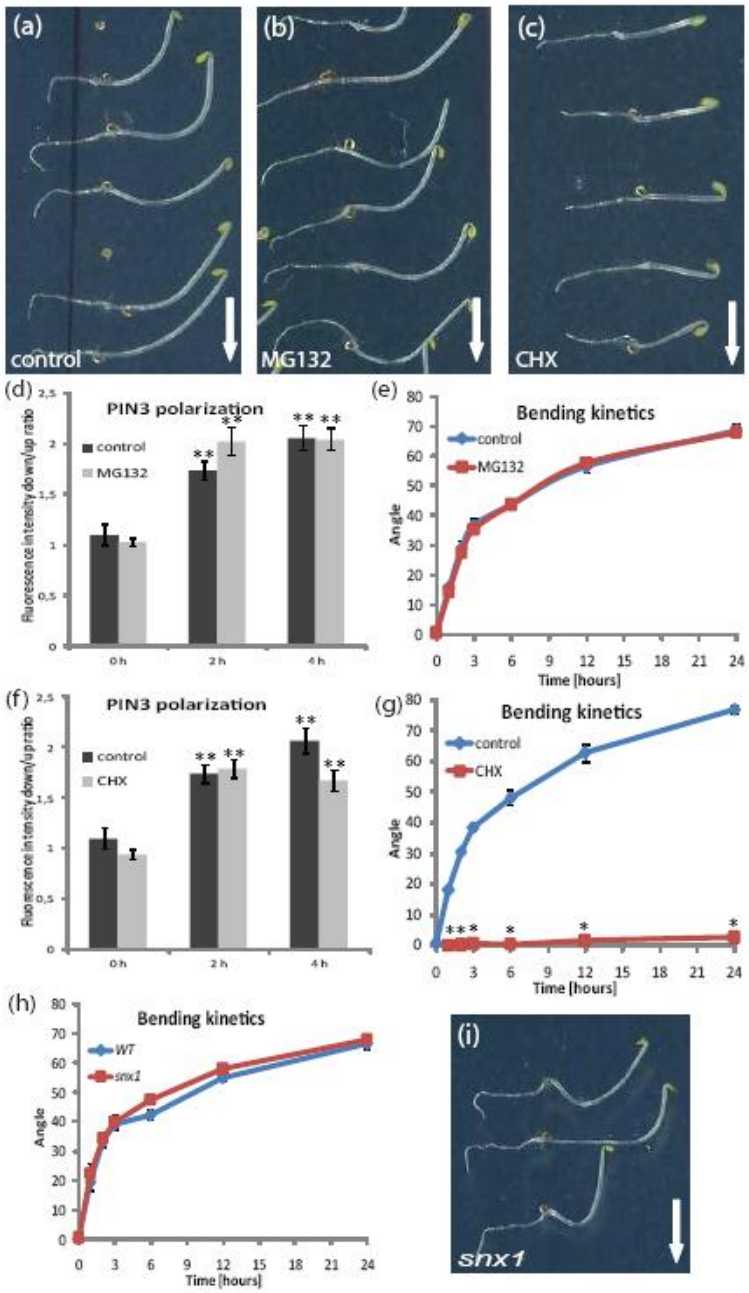

Figure S1. Involvement of degradation and *de novo* biosynthesis in hypocotyl gravitropic response.
(a-c) Bending of wild-type hypocotyls on mock plates (a), supplemented with 25 µM MG132 (b) or supplemented with 50 µM CHX (c) after 1 hour pre-treatment and 24 hours of gravistimulation.
(d-g) Gravity-induced PIN3-GFP polarization in the hypocotyl endodermis on mock plates or plates supplemented with 25 µM MG132 (d) or 50 µM CHX (f). Values are the average of three biological experiments (n>10 per time point on each replicate). Error bars are SE (Student's *t* test: ***P*<0,0001). Hypocotyl bending kinetics of the wild type on plates supplemented with 25 µ MG132 show response (e) (Student's t test between mock and MG132 treated show no significant differences). Hypocotyl gravitropic response is strongly reduced by 50 µM CHX (g) (Student's *t* test: **P*<0.0001). (h and i) Similar bending of wild-type and *snx1* hypocotyls after 24 hours of gravistimulation (i). Bending kinetics of wild type and *snx1* mutant hypocotyls (h). Error bars are SE. Arrows mark gravity direction. Values are the average of three biological replicates (n>10 per time point on each replicate). Error bars are SE (Student's *t* test show no significant differences).

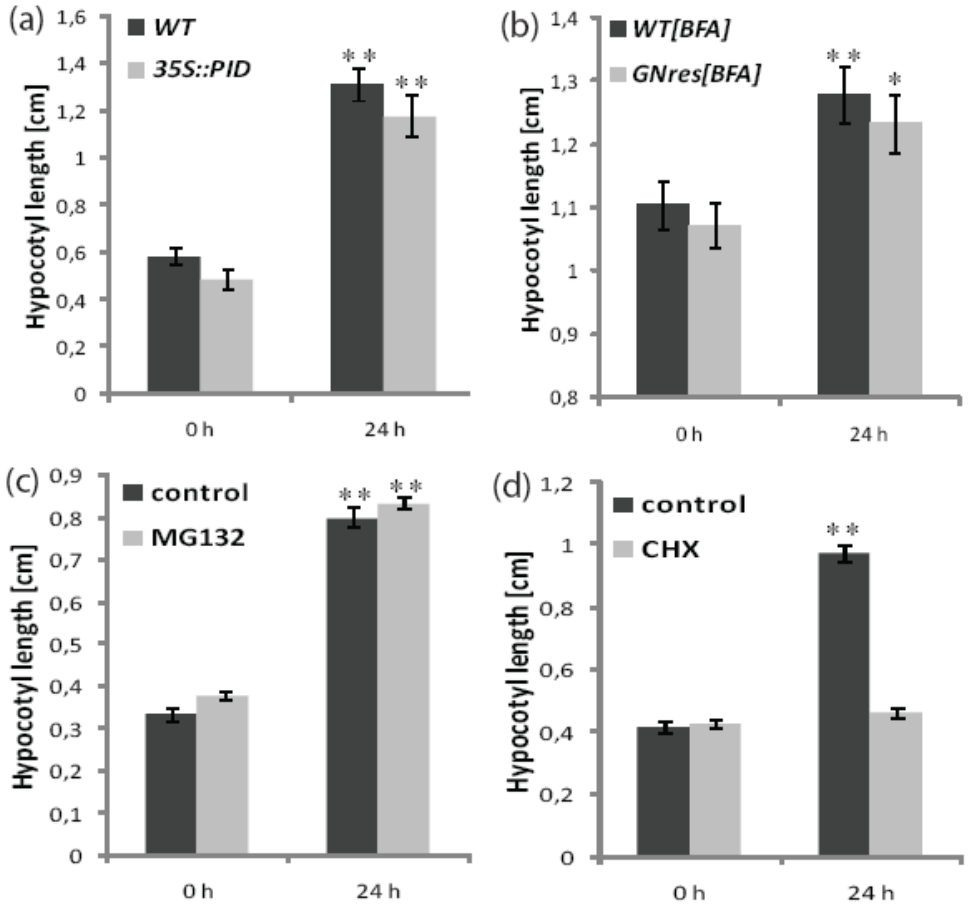

Figure S2. Hypocotyl growth in used genotypes and following treatments.
(a-d) Hypocotyl growth of wild-type and *35S::PID* (a), wild-type and BFA-resistant *GNOM*^{M696L} line on BFA (b), wild type on mock plates or supplemented with 25 µM MG132 (c) or 50 µM cycloheximide (d). Hypocotyl length was measured before stimulation and after 1 hour pre-treatment following 24 hours of gravistimulation and average lengths were calculated. Error bars are SE. Values are the average of three biological experiments (n>10 per time point on each replicate). Error bars are SE (Student's *t* tests: **P*<0,008; ***P*<0,0001).

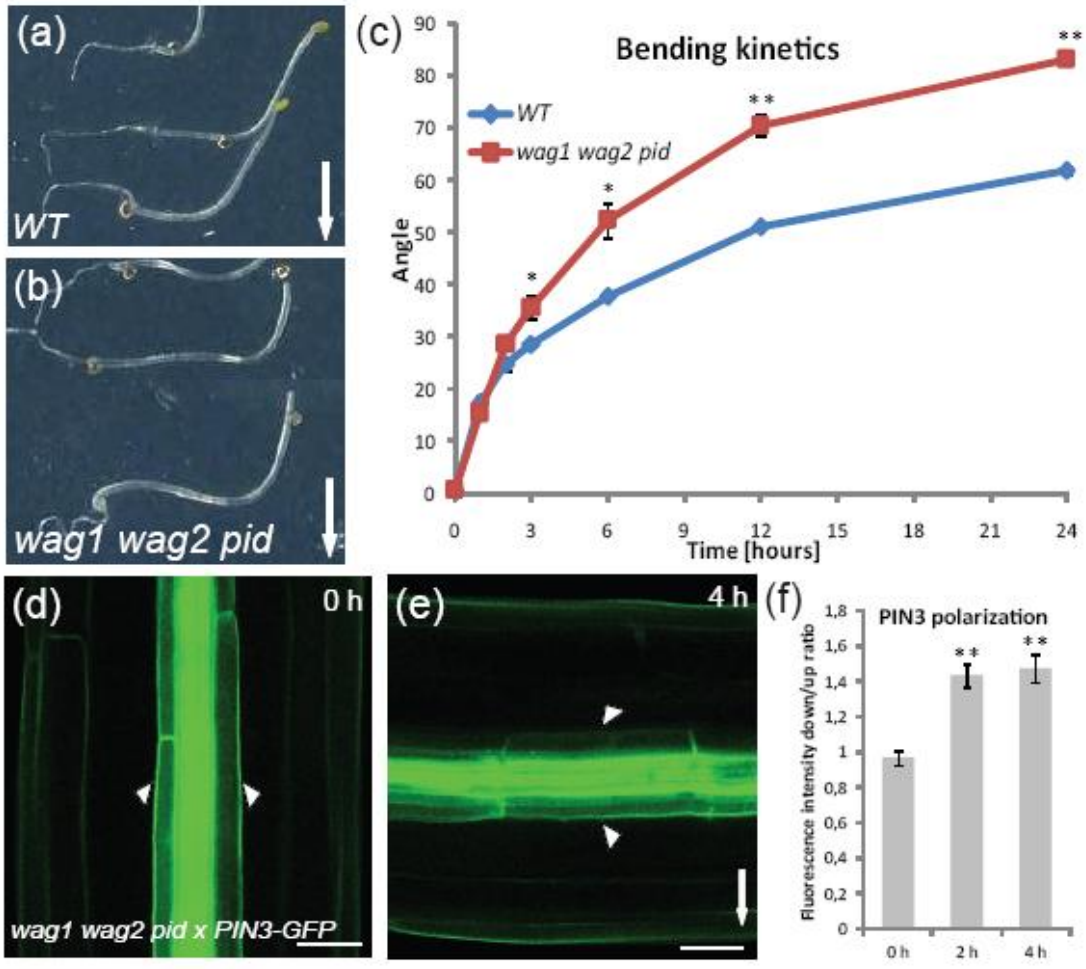

Figure S3. PINOID requirement for PIN3 polarization.
(a-c) Hypergravitropic response of *wag1 wag2 pid* triple-mutant hypocotyls (b) after 24 hours of reorientation as compared to wild type (a). Kinetics of hypocotyl bending: angles were measured after 1, 2, 3, 6, 12, 24 hours and average curvatures were calculated (c). Values are the average of three biological replicates (n>10 per time point on each replicate). Error bars are SE (Student's t test: *P<0.05, **P<0.005).
(d-f) Gravity-induced PIN3-GFP polarization in the *wag1 wag2 pid* hypocotyls after 4 hours of gravistimulation (e). Non-stimulated *wag1 wag2 pid* control (d). Scale bar: 50 μm. Ratio of PIN3-GFP fluorescence intensities at the outer side of endodermis cells at the lower versus upper hypocotyl side after different times of gravistimulation (f). Values are the average of one biological experiment (n>10 per time point on each replicate). Error bars are SE (Student's *t* test: ***P*<0.0001).

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Chapter 3.

Auxin feed-back on PIN3 polarity for termination of shoot tropic responses

Adapted from

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HR and JF initiated the project and designed most of the experiments, HR carried out the experiments. HR, HSR and FJ discussed the experimental setup and procedures. HR wrote the manuscript and HSR and JF assisted manuscript writing.

Auxin feed-back on PIN3 polarity for termination of shoot tropic responses

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Abstract

Tropisms are adaptation responses, through which plants modify their growth in response to signals such as light or gravity. It involves perception and asymmetric distribution of the plant hormone auxin. Here we identify a crucial part of the gravitropic mechanism, an auxin feed-back for termination of hypocotyl tropic responses. We show that at later stages of hypocotyl bending, auxin polarizes the localization of PIN auxin efflux carriers in hypocotyl endodermal cells, reestablishing auxin symmetry and terminating differential growth. In response to auxin asymmetry PIN3 relocates to the inner membranes, depleting auxin from the bending site and preventing an overbending response. Thus, dynamic PIN3 relocalization at different time points of the gravity response presumably redirects auxin flow first towards the lower side, where auxin promotes growth, causing hypocotyls to bend with gravity vector, than depletes the auxin maximum to terminate the bending. Our results imply that auxin-mediated recruitment of PIN3 into distinct trafficking pathways is a mechanism to polarize auxin fluxes in response to different environmental and endogenous cues.

Introduction

Tropism is way for plants to adapt their growth to environmental changes such as light direction (phototropism) and gravity (gravitropism). Gravitropism is the directed growth of plants with respect to the gravity vector. Roots grow with the gravity direction – positive gravitropism, while shoots grow against gravity vector – negative gravitropism. Gravitropism consists of several stages, including perception of the gravity stimuli, signal transduction, asymmetric growth response resulting in organ bending during which the curvature of the organ changes over time until a set-angle and a steady-state shape is reached (Bastien et al., 2013). Gravitropism involves the asymmetric distribution of the plant-specific signaling molecule auxin (Friml et al., 2002). Higher auxin content in the cells at the lower side of the hypocotyl stimulates cell elongation resulting in differential growth and bending of the hypocotyl against the gravity vector.

In hypocotyl the gravity sensing takes place in endodermal cells, where heavy organelles sediment along the gravity vector (Hashiguchi et al., 2013; Morita, 2010). The hypocotyl endodermis constitutes a barrier between the main basipetal auxin flow in the vasculature and the outer cell layers where the auxin-dependent control of elongation occurs (Gendreau et al., 1997). The asymmetry in auxin distribution during tropism responses is generated by polar transport mediated predominantly by the PIN3 auxin efflux carrier (Ding et al., 2011; Friml et al., 2002; Rakusová et al., 2011). Gravity stimulation polarizes PIN3 to the lower sides of hypocotyl endodermal cells or root columella cells which correlates with increased auxin accumulation in adjacent tissues. Subsequently auxin induces the differential growth resulting in bending of the organ. A curvature response is established that allows the hypocotyl to resume growth at a defined set angle with the gravity vector (Masson et al., 2002). The signal inducing hypocotyl bending has to be at specified point terminated and the auxin symmetry should be restored to prevent overbending response. There is many works focused on tropic bending initiation, but termination issue is often neglected.

In other developmental contexts, auxin acts as a polarizing signal, as it moves between cells in a directional manner. This directionality is determined by the polarized localization of PINs (Petrášek et al., 2006) in the plasma membrane (PM) of individual cells (Wiśniewska et al., 2006). Therefore changes in PIN localization are needed to provide immediate alteration in auxin transport. Furthermore, feed-back regulation of PIN polarization by auxin is one of the mechanisms proposed to regulate plant development such as vascular tissue differentiation and regeneration (Sauer et al., 2006) and during embryogenesis (Wabnik et al., 2013). This feed-

back would be the underlying mechanism for a coordinated and self-organizing polarization of individual cells, ultimately leading to tissue polarity (Prusinkiewicz and Runions, 2012). Indeed, changes in PIN polar localization in response to auxin have been observed (Balla et al., 2011; Sauer et al., 2006), but similar auxin feed-back in context of tropisms has not been indicated.

The auxin effect on PIN polarity seems to require both known auxin signaling pathways. Auxin signaling leading to transcriptional response involves the $SCF^{TIR1/AFB}$-mediated proteolysis of the Aux/IAA family of transcriptional regulators with the TRANSPORT INHIBITOR RESPONSE1 (TIR1)/AUXIN SIGNALING F-BOX (AFB) proteins functioning as auxin receptors (Chapman and Estelle, 2009). In addition, Auxin Binding Protein 1 (ABP1) (Jones and Herman, 1993; Grones and Friml, 2014) is another auxin receptor implicated in non-transcriptional auxin responses (Robert et al., 2010; Chen et al., 2014).

Here we identified a mechanism how the tropic bending is terminated. The initially established auxin asymmetry may feed-back on PIN3 repolarization and further reestablishment of auxin symmetry. This may terminate differential cell elongation and terminate bending response. This phenomenon involves feed-back between auxin distribution and PIN3 intracellular localization as well as protein stability.

Results

Auxin distribution and PIN3 polarization during gravity response of Arabidopsis hypocotyl

We analyzed in detail the hypocotyl gravitropic response by kinetic analyses of the bending. An initial gravity bending response was observed after 3h of 90° gravi-stimulation. After approx. 19h of gravi-stimulation bending response slowed, later the bending stopped (Figure 1a; marked with orange) and hypocotyl grew straight upward (Figure 1a). To describe in more detail what happens in hypocotyls during different stages of the bending response, we monitored the asymmetric auxin response, based on the expression pattern of the auxin reporter *DR5rev::GFP* (Friml et al., 2003) and changes in PIN3-GFP (Žádníková et al., 2010) localization at different time points following gravi-stimulation. We focused on the bending part of hypocotyl. Scoring *DR5rev::GFP* expression, we detected pronounced signal asymmetry after 6h of gravi-stimulation (Figure 1b and 1d). Approximately after 36h of stimulation *DR5rev::GFP* showed no more asymmetric GFP signal (Figure 1b and 1e) (See in Figure S1 and Experimental Procedures for detailed description and statistic evaluation). PIN3-GFP showed apolar PM localization in endodermal cells in dark-grown hypocotyls (Figure 1b and 1f). As described earlier (Rakusová et al., 2011) we observed that PIN3-GFP relocate to the lower side of the endodermal cells as soon as after 2h of gravi-stimulation and maintains the localization (Figure 1b and 1g). After 18h of stimulation, we noticed that PIN3-GFP at the lower side of the hypocotyl polarized from the outer side and remained only in the inner-lateral PM of the endodermis (Figure 1b and 1h). In summary, we observed dynamic changes in PIN3-GFP localization and auxin response. First the symmetric PIN3 localization reestablish to the lower side of endodermal cells followed by auxin response accumulation at the lower hypocotyl side. Later PIN3 relocates from lower endodermal cell side to the inner side; this correlated with re-established symmetric auxin response and followed by hypocotyl upward growth.

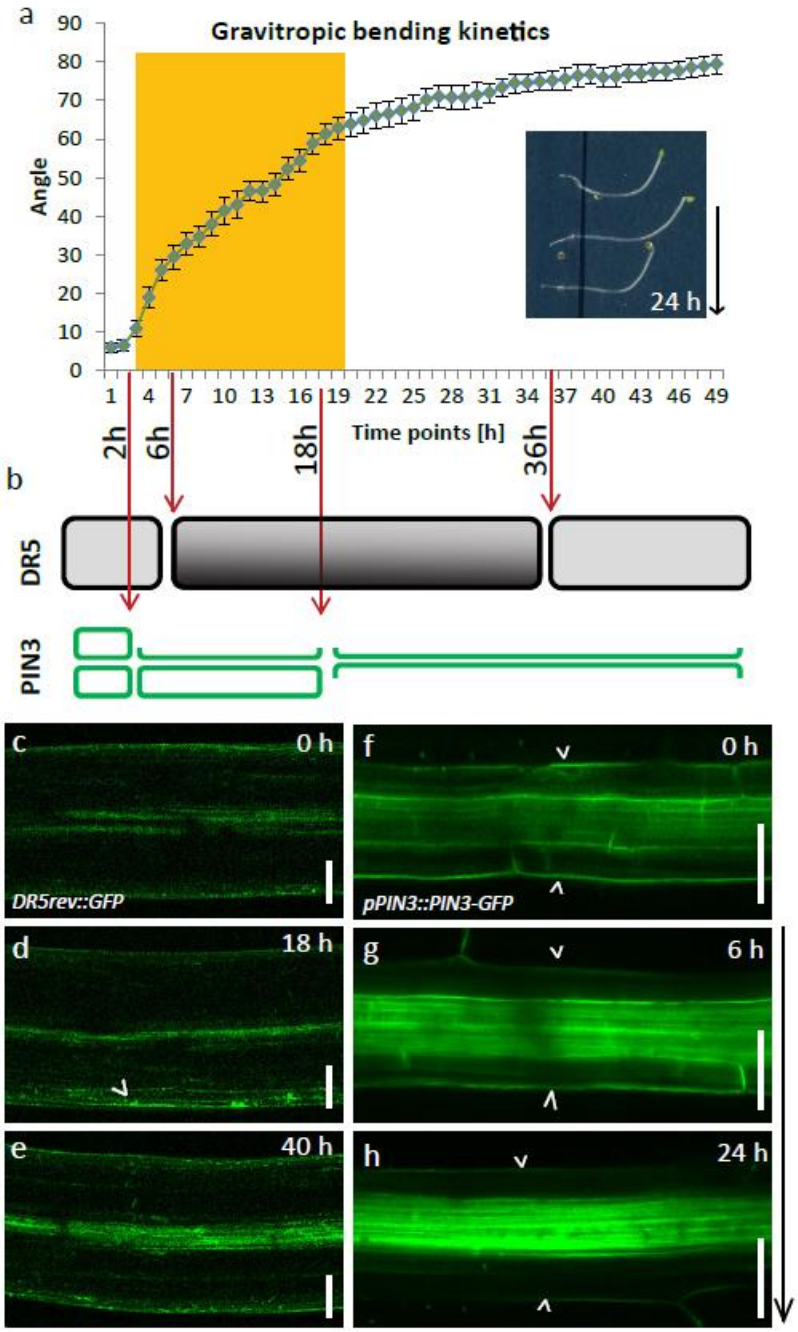

Figure 1. Changes in PIN3 localization and *DR5rev::GFP* accumulation during gravitropic bending response of *Arabidopsis* hypocotyl

(a) Hypocotyl bending kinetics of wild type hypocotyls during gravitropic response. Hypocotyl curvatures were measured every hour and average curvatures were calculated. Values are the average of three biological replicates (n > 10 per time point on each replicate). Error bars represent SE. Orange marks gravity bending response.

(b) Scheme summarizing *DR5rev::GFP* accumulation changes and PIN3 localization in dark grown hypocotyl upper and lower endodermis cells excluding stele during different time points of gravity response of hypocotyl.

(c-e) Establishment of *DR5rev::GFP* activity in hypocotyls after 0h (c), 18h (d) and 40h (e) of gravity stimulation. *DR5rev::GFP* showed uniform expression pattern in the hypocotyl without any stimulation (c). After 6 hours of gravity stimulation *DR5rev::GFP* accumulated in the lower side of hypocotyl (picture shows *DR5rev::GFP* after 18h of stimulation, d). This accumulation vanished after 32-36h after the gravity stimulation (picture shows *DR5rev::GFP* after 40h of stimulation, e). Arrowhead points to *DR5rev::GFP* accumulation.

Arrows indicate gravity direction. Scale bars represent 50 µm.

(f-h) PIN3-GFP localization in endodermis cells of hypocotyls after 0h (f), 6h (g) and 24h (h) of gravity stimulation. PIN3-GFP was apolarly localized in the hypocotyl endodermal cells before gravity stimulation (f). Following gravistimulation, PIN3-GFP localized at the lower side of endodermal cells of the upper hypocotyl side between 2 and 18 hours after stimulation (picture shows PIN3-GFP after 6h stimulation, g). After prolonged

stimulation, PIN3 relocalized from the outer-lateral side of lower side of hypocotyl (picture shows PIN3-GFP after 24h stimulation, h). Arrowheads point outer PM of endodermal cells. For experiment setup and quantification see Figure S1.

Feed-back regulation of PIN3 polarization by auxin

Next we addressed how the unexpected change of PIN3 localization to the inner side at later stages of gravitropic response occurs. The attractive hypothesis assumes that auxin accumulation at the lower site influences the PIN3 localization. To test if auxin itself causes some changes in PIN3-GFP localization, etiolated seedlings were treated with natural -10 µM IAA (Indole-3-acetic acid) and synthetic auxin -NAA (Naphthalene-1-acetic acid) for 4h. Indeed, auxin treatment induced change of PIN3-GFP localization to the inner side of endodermal cell in hypocotyl – "inner-lateralization" (Figure 2a-2d; arrowheads indicate loss of PIN3-GFP signal at outer-lateral cell sides). Measuring the ratio of PIN3-GFP fluorescence intensity between the inner lateral and the outer lateral endodermal PM allowed us to assess the concentration- and time-dependence of this auxin effect. PIN3-GFP relocation after IAA treatment was significant after 3h (Student's t-test; $P < 0,01$) and more pronounced after 4h ($P < 0,001$) and 6h ($P < 0,001$) of treatment. Already 1 µM IAA led to a significant relocation ($P < 0,05$), which was better visible at 10 µM ($P < 0,001$) (Figures 2e and 2f). NAA causes significant PIN3 inner-lateralization after 2 h ($P < 0,05$) and more pronounced after 4h ($P < 0,001$) of treatment (Fig S2a, S2b). No further increase in this effect was observed after 4h. As for IAA, 1 µM NAA led to a significant PIN3 relocation ($P < 0,05$), which was even more pronounced at 10 µM ($P < 0,001$) (Figures S2a and S2b). In all following experiments described below, we used 10 µM of NAA for 4 h as standard auxin treatment. Similar to exogenous auxin applications, higher endogenous auxin content in hypocotyls of *yucca1D* (Zhao et al., 2001) a dominant gain-of-function mutant in a key regulator of auxin biosynthesis, was sufficient to trigger inner-lateralization of PIN3-GFP (Figure 2g-2i). Notably, despite PIN3 is to large extent inner-lateralized in *yucca1D*, it still show the gravity-induced relocation (Figure S2d, S2e) and only slower bending response (Figure S2c).

Next, we examined whether the auxin effect on PIN localization is specific to PIN3. To test this we used *PIN7::PIN7-GFP* line, since PIN7-GFP shows the hypocotyl expression and localization similar to PIN3-GFP, and *SCR::PIN2-GFP* to express PIN2 ectopically in hypocotyl endodermis. After auxin treatment, PIN7-GFP and PIN2-GFP relocated to inner-lateral side (Figure S3a, S3g, S3i), similarly to PIN3-(Y)GFP (Figure S3a, S3f, S3h). This indicates that auxin effect is not specific to a particular PIN protein.

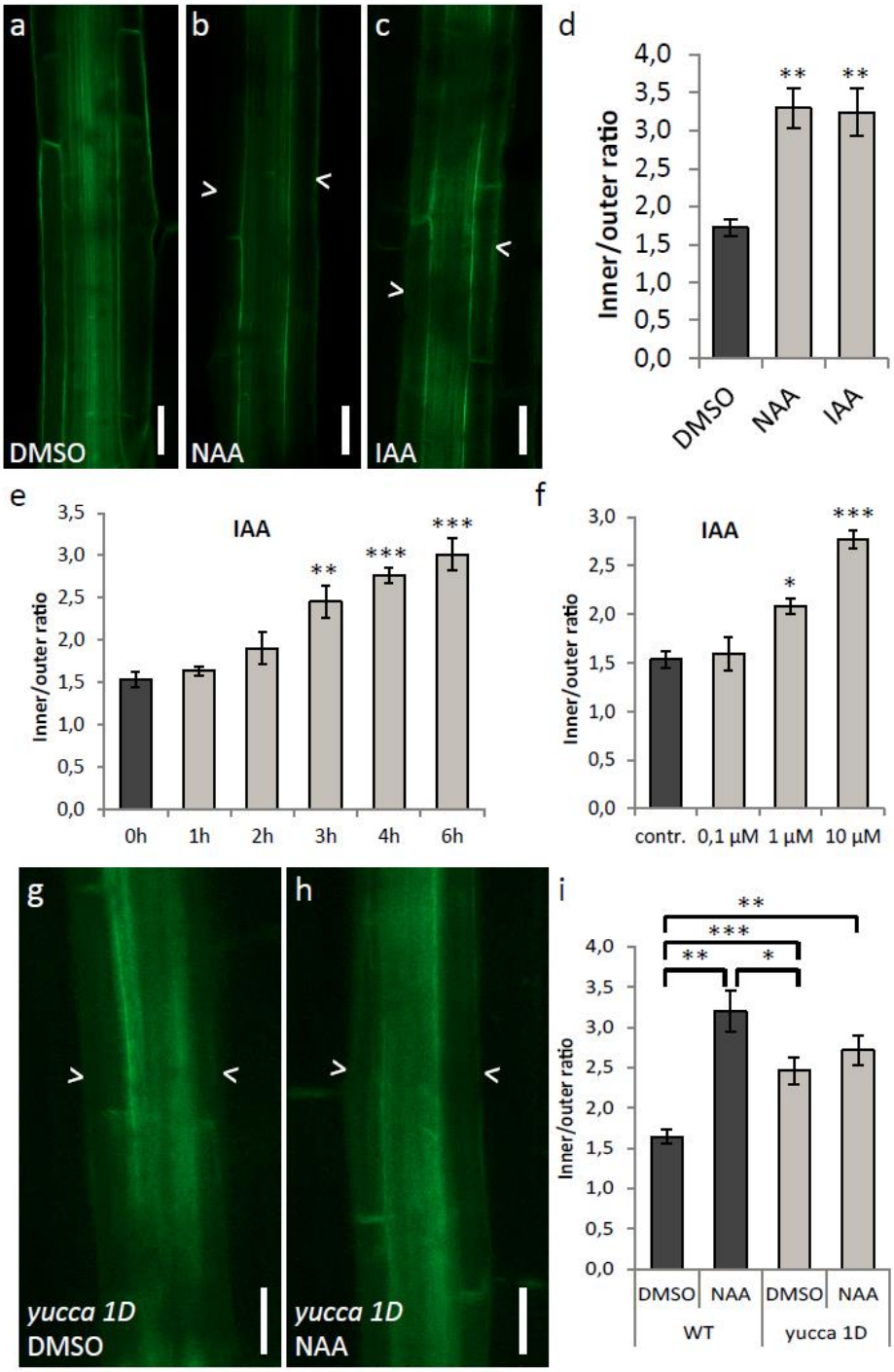

Figure 2. Auxin-induced PIN3 relocation from the outer side of endodermal cells in hypocotyl

(a-d) Auxin caused PIN3-GFP relocation from outer side to inner side of endodermal cells. After 4h of 10 µM NAA (b) and 10 µM IAA (c) treatment PIN3-GFP relocated to the inner-lateral side compared to control situation (a). Arrowheads point PIN3-GFP depletion from outer lateral cell side. (d) Quantitative evaluation of auxin-dependent PIN3 relocation in endodermal cells.

(e, f) Time (e) and concentration (f) dependence of auxin effect using 10 µM IAA for different time points or 4 hours treatments with different IAA concentrations, respectively. Graphs show mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, * $P < 0,05$; ** $P < 0,01$; *** $P < 0,001$).

(g-i) Auxin-overproducing mutant *yucca1D* showed depletion of PIN3-GFP from outer side of endodermal cell in hypocotyl in control condition (g) and after auxin treatment (h). (i) Graphs show mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, * $P < 0,05$; ** $P < 0,01$; *** $P < 0,001$).

Arrowheads point to PIN3-GFP depletion from outer lateral side of endodermal cells. Scale bars represent 20 µm.

Thus, higher auxin levels resulting from exogenous application or endogenous production induced inner-lateralization of PIN3 in hypocotyl endodermal cells providing possible mechanism for re-establishment of symmetric PIN3 localization at the later stages of hypocotyl tropic responses. This tool (auxin treatment) was used to identify the molecular components involved in PIN3 localization as compared to the faster mechanism in response to gravity stimulation.

Clathrin-mediated endocytosis and GNOM-dependent recycling for auxin-induced PIN3 relocation

PIN proteins constitutively cycle between the PM and endosomal compartments (Geldner et al., 2001). This subcellular dynamism requires clathrin-mediated endocytosis (Dhonukshe et al., 2007; Kitakura et al., 2011) and GNOM ARF GEF dependent recycling (Geldner et al., 2003).

To test a role of clathrin during the PIN3 relocation, we used Tyrphostin23 (Tyr23), a drug interfering with clathrin-mediated PIN endocytosis (Dhonukshe et al., 2007) Tyr23 blocked both gravity- and auxin-mediated PIN3-GFP relocation in endodermal cell (Figure S4a-S4h). In addition, we tested conditionally overexpressing the C-terminal part of the clathrin heavy chain (termed HUB1) that exerts a dominant negative effect on clathrin function (*pINTAM>>RFP-HUB1;* Liu et al., 1995). This interference with the clathrin function inhibited PIN3-GFP gravity-induced relocation (Figure S4i-S4k) as well as auxin-induced PIN3-GFP inner-lateralization (Figure 3a-3e). These observations suggest a role of clathrin-mediated endocytosis during both processes.

Next we addressed whether auxin-induced PIN3 relocation requires GNOM-dependent vesicular trafficking as was showed previously to gravity-induced PIN3 relocation (Rakusová et al., 2011). When seedlings were treated with Brefeldin A (BFA), a fungal drug inhibiting a subclass of ARF–GEF including GNOM, then co-treated with BFA/NAA, PIN3-GFP remained at the outer PM, and no inner-lateralization was detected (Figure 3f-3h). The inhibitory effect of BFA on auxin-induced PIN3-GFP inner-lateralization was partially rescued in a transgenic line carrying a version of GNOM protein resistant to BFA ($GNOM^{M696L}$; (Geldner et al., 2003) Figure S4l-S4o) confirming GNOM requirement for this process. These experiments suggest the importance of clathrin and GNOM ARF GEF for both gravity-induced PIN3 polarization (Rakusová et al., 2011) and also auxin-induced PIN3 inner-lateralization.

Vacuolar targeting and protein degradation for auxin-induced PIN3 relocation

Separate measurements of PIN3-GFP fluorescence intensity in the outer and inner lateral side revealed that during gravity-induced PIN3-GFP relocation, PIN3-GFP signal decreases from upper side and increases in the lower side of the cell. In contrast, after auxin treatment the signal in the outer lateral side decreased, but did not increase in the inner-lateral side (Figure S6k). This would suggest contribution of protein degradation during the auxin-mediated PIN3 relocation compared to gravity-induced PIN3 relocation.

For this reason we tested contributions of protein synthesis and protein degradation to auxin-induced PIN3 inner-lateralization. We analyzed first the requirement of protein synthesis by blocking it with its inhibitor cycloheximide (CHX) with or without co-treatment with NAA. PIN3-GFP was inner-lateralized following CHX/NAA co-application (Figure S5a-S5c). This data indicates that auxin effect on PIN3 relocation does not depend on *de novo* protein synthesis. Interestingly, PIN3-GFP inner-lateralization was even more pronounced after CHX co-treatment suggesting, that PIN3 abundance in general might play significant role (Figure S5c) – less PIN3 in the PM, more polar localization restricted to inner-lateral cell side.

The treatment with MG132, a drug blocking proteasome function and PIN degradation (Abas et al., 2006), does not affect PIN3 gravity-induced relocation during hypocotyl gravitropism (Rakusová et al., 2011). Nevertheless, we tested if protein degradation would be involved in auxin-induced PIN3 inner-lateralization. Indeed, MG132 treatment reduced the auxin effect on PIN3 relocation (Figure 3i, Figure S5d and S5e), indicating that protein degradation contributes to auxin-induced PIN3 inner-lateralization.

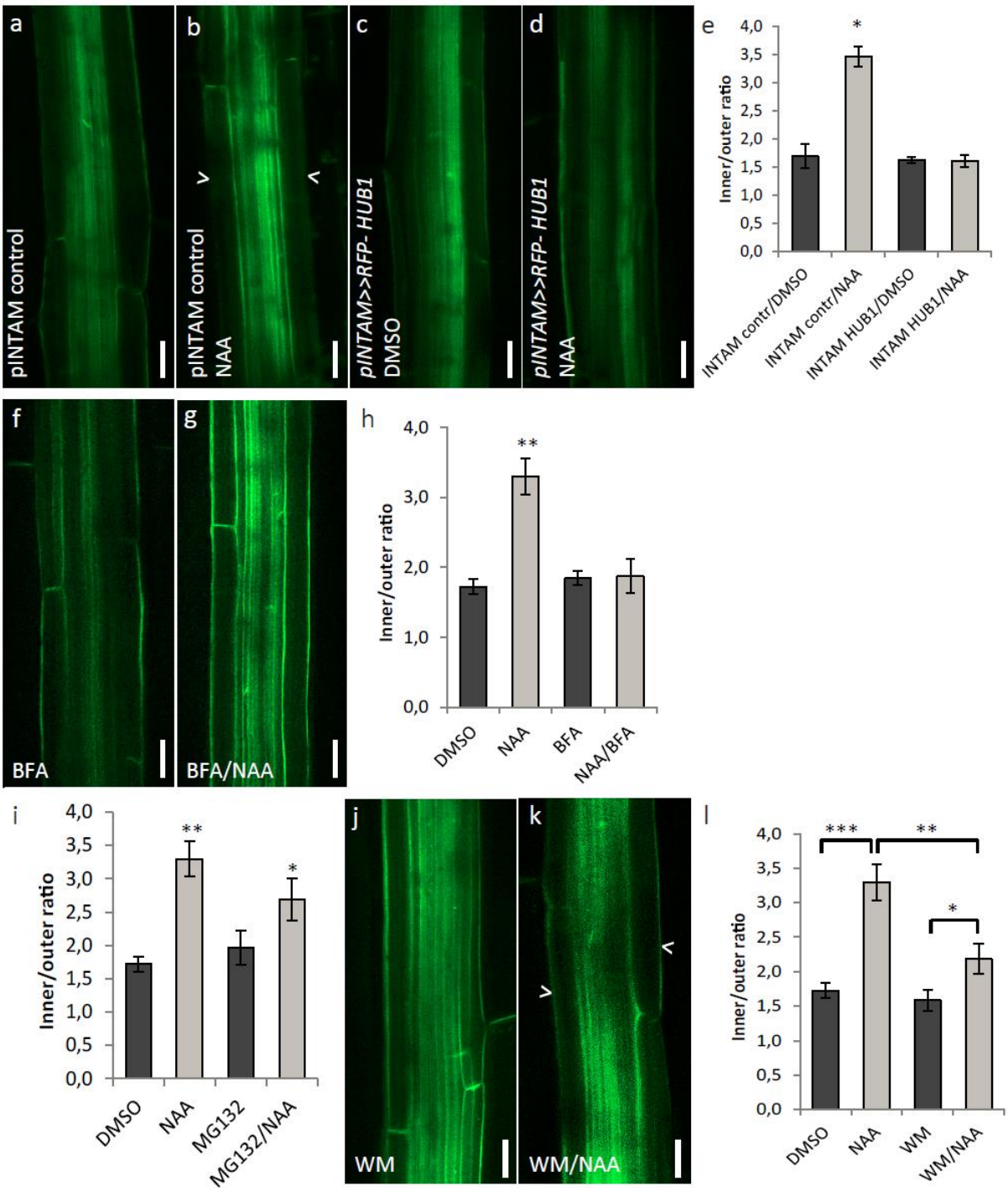

Figure 3. Auxin-induced PIN3 relocation requires clathrin- and GNOM-mediated endocytosis and depends on protein degradation and vacuolar targeting

(a-e) No altered PIN3-GFP localization in induced *pINTAM* control (a) and induced *pINTAM>>RFP-HUB1* line (c). Induced *pINTAM>>RFP-HUB1* showed inhibition of auxin effect on PIN3-GFP inner-lateralization (d) compared to the control (b). (e) Quantitative evaluation of auxin-dependent PIN3-GFP relocation in endodermis cells. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, * $P < 0,05$). Arrowheads point PIN3-GFP depletion from outer lateral side of endodermal cells.

(f-h) Brefeldin A (BFA) affected auxin-induced PIN3-GFP relocation in hypocotyl endodermal cells (f, 5h BFA alone; g, 1h pre-treatment followed 4h co-treatment with NAA). (h) Ratio of PIN3-GFP fluorescence intensity at the outer and inner side of endodermis cells at hypocotyl after BFA treatment or BFA/NAA co-treatment. Error bars are SEs (Student's t-test, ** $P < 0,01$).

(i) MG132 co-treatment showed reduction of PIN3-GFP inner-lateralization after NAA treatment. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, * $P < 0,05$; ** $P < 0,001$). See also Figure S6D, S6E.

(j-l) Auxin-induced PIN3-GFP relocation was partially inhibited by wortmanin (WM)/NAA co-treatment (k) compared to control treated with WM alone (j). (l) Quantitative evaluation of auxin-dependent PIN3-GFP relocation in endodermis cells. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, * $P < 0,05$; ** $P < 0,01$; *** $P < 0,001$).
Arrowheads point to PIN3-GFP depletion from outer lateral side of endodermal cells. Scale bars represent 20 µm.

Auxin was shown to control PIN abundance at the PM by promoting their vacuolar targeting and degradation as part of the gravitropic response in root (Baster et al., 2013; Kleine-Vehn et al., 2008). To further characterize if protein degradation via vacuolar targeting is needed for PIN3 relocation, we tested the effect of wortmanin (WM), an inhibitor of the PIN trafficking to the vacuole (Kleine-Vehn et al., 2008). WM did not influence gravity bending response (Figure S5g) or gravity-induced PIN3-GFP relocation to the lower side of endodermal cell (Figure S5h-S5j). However, WM treatment interfered with PIN3-GFP relocation after auxin co-treatment as manifested by persistent PIN3-GFP localization at the outer endodermis cell sides. Effect of auxin was not completely blocked, but reduced ($P > 0,05$) by WM co-treatment (Figure 3j-3l).

These data indicates the role of PIN3 abundance (protein synthesis and degradation) during the auxin-mediated change of PIN3 localization.

PINOID-mediated phosphoregulation is required for auxin-induced PIN3 relocation

PIN phosphorylation by the serine/threonine protein kinase PINOID (PID) is one of the key PIN polarity regulation (Friml et al., 2004; Huang et al., 2010; Zhang et al., 2010). We tested whether phosphorylation by PID contributes to auxin-mediated PIN3 inner-lateralization as it was shown in case of gravity-induced PIN3 relocation (Rakusová et al., 2011). In this case we used the strong *PID* overexpressor line *35S::PID-21* (Benjamins et al., 2001). *35S::PID-21* was resistant to auxin-mediated PIN3-GFP inner-lateralization (Figure 4a-4c). Furthermore, we tested *wag1 wag2 pid* triple mutant, lacking activity of PID and its two closest homologues WAG1 and WAG2 (Santner and Watson, 2006). The triple *wag1 wag2 pid* mutant showed more inner-lateral PIN3-GFP localization regardless of auxin treatment (Figure 4d and 4f) and inner-lateralization of PIN3-GFP in *wag1 wag2 pid* mutant was only slightly more pronounced after additional auxin application (Figure 4e and 4f). Taken together, PID-dependent phosphorylation is required for PIN3 targeting to the outer membrane and dephosphorylation is needed for auxin-dependent PIN3 inner-lateralization.

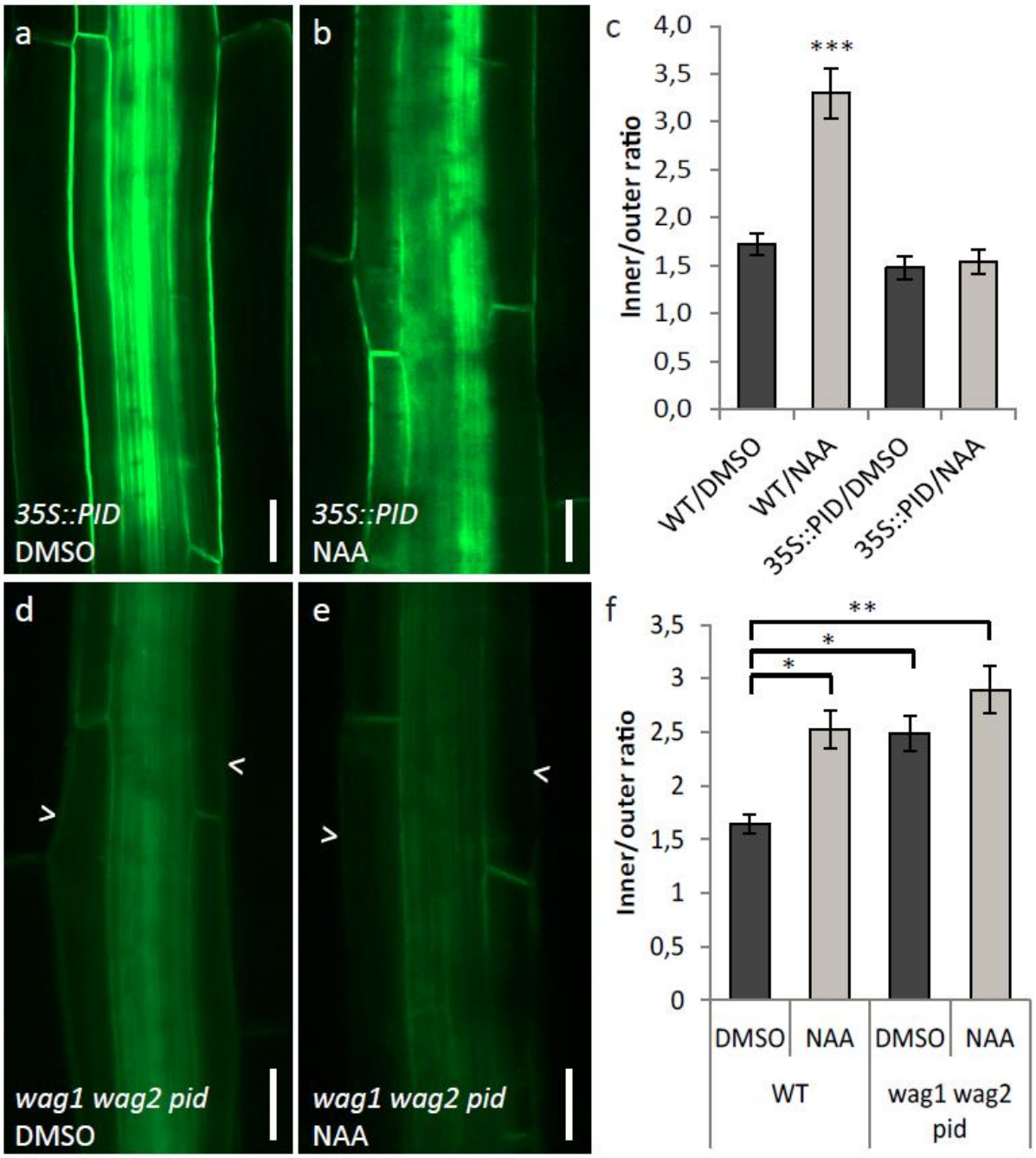

Figure 4. PINOID-mediated phosphorylation is required for auxin-induced PIN3 inner-lateralization
(a-b) Overexpression of PINOID protein kinase in *35S::PID* led to inhibition of PIN3-GFP auxin-induced inner-lateralization (b) in hypocotyls compared with non-treated controls (a). (c) Quantitative evaluation of auxin-dependent PIN3-GFP relocation in endodermis cells. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, *** $P < 0,001$).
(d-e) Inner-lateralized PIN3-GFP in *wag1 wag2 pid* mutant (d) is not affected by slightly more pronounced after NAA treatment (e). (f) Quantitative evaluation of auxin-dependent PIN3-GFP relocation in endodermis cells. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, * $P < 0,05$; ** $P < 0,01$).
Arrowheads point PIN3 depletion from outer lateral side of endodermal cells. Scale bars represent 20 µm.

ABP1 and TIR1 signaling pathway for auxin-induced PIN3 relocation

So far two auxin signaling mechanisms are known, namely ABP1 and TIR1 (Hayashi, 2012). To test whether auxin regulation of PIN3 inner-lateralization requires ABP1- and/or

TIR1-dependent signaling pathways, we took the opportunity of structural analogs of the natural auxin (IAA) to uncouple the two activities of IAA; the TIR1-dependent induction of gene expression and the ABP1-dependent inhibition of PIN internalization (Robert et al., 2010; Simon et al., 2013). For instance, auxin analogs a-(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA) (Hayashi et al., 2008) and auxinole (Hayashi et al., 2012) specifically target ABP1-dependent inhibition of PIN internalization, without inducing the expression of auxin-inducible reporters such as DR5, specific to the TIR1-dependent signaling pathway. PEO-IAA and auxinole can bind TIR1/AFB proteins similarly to endogenous IAA, but cannot promote the interaction with the (AUX)/IAA proteins and their consequent ubiquitination and degradation. In contrast, another auxin analog, 5-fluoroindole-3-acetic acid (5-F-IAA) activated *DR5rev::GFP* expression but failed to inhibit ABP1-mediated PIN internalization (Robert et al., 2010) (Figure S6a). Using these auxin-like compounds we tested which auxin signaling pathway is used for mediating auxin-dependent PIN3 relocation. PEO-IAA (Figure S6b and S6e) and auxinole (Figure S6b and S6f) relocated PIN3-GFP after 4h to the similar extent as NAA (Figure S6b and S6d). By contrast, 5F-IAA had not such effect and did not affect the apolar localization of PIN3-GFP (Figure S6b and S6g), similarly to negative control treatment with Naphthalene (Figure S6b and S6c). These results suggest that auxin-induced PIN3 inner-lateralization does not strictly require TIR1-dependent signaling, but relies on ABP1-dependent inhibition of PIN endocytosis.

To confirm these observations, we tested mutants impaired in auxin perception (ABP1 and TIR1 auxin receptors), for the effects of gravity and auxin on PIN3 relocalization. We investigated the potential role of ABP1 using *35S::ABP1* gain-of-function line (Rakusová and Wabnik, unpublished), *abp1* knock-down lines (*ABP1* antisense (*abp1 AS*), *SS12S6* and *SS12K9*; (Braun et al., 2008)) and the *abp1-5* mutant, containing a histidine replacement in the ABP1 auxin binding site (Robert et al., 2010; Xu et al., 2010). Plants expressing *35S::ABP1* showed no altered PIN3-GFP relocation neither after gravity stimulation nor after auxin application (Figure S6h-S6l). Conversely, *abp1* mutants differentially responded to gravity- and auxin-induced PIN3 relocation. Gravity bending kinetics of *abp1* knock-down lines was altered with a very inconsistent and variable behavior: several hypocotyls bended opposite of the gravity vector, several overbended (Figure 5a and 5b). Interestingly we didn't detect any defect in gravity-dependent PIN3-GFP relocation in any of the mutants (Figure 5c-5f). Quantification of the auxin-dependent lateralization of PIN3-GFP in endodermal cells in the *abp1* knock-down lines illustrated an inner-to-outer asymmetry in PIN3 localization, with PIN3 accumulating at the inner membranes regardless of the auxin treatment (Figure 5j-5k). Furthermore, auxin

treatment had no additional effects (Figure 5j and 5l). To confirm these data we used *abp1-5* line, which localized PIN3-GFP as wild type in a non-stimulated conditions (Figure 5g and 5i) and after gravity-stimulation (Figure 5e and 5f) but fail to relocate PIN3-GFP after auxin application (Figure 5g-5i).

Similarly we investigated the involvement of the TIR1 signaling pathway by testing *tir1 afb2 afb3* triple mutant (Dharmasiri et al., 2005) seedlings for PIN3 relocation in the given conditions. The *tir1 afb2 afb3* exhibited normal gravity-induced PIN3-GFP polarization (Figure S7a, S7b). In contrast to *abp1* mutants, *tir1 afb2 afb3* displayed normal PIN3-GFP inner-lateralization induced by auxin (Figure S7c-e).

In summary, these data show that, gravity-induced PIN3 PM relocation during the first stage of gravity response does not require auxin signaling. In contrast, the auxin effect on PIN3 relocation at latter stages involves the ABP1-dependent auxin signaling but not strictly the TIR1-dependent auxin signaling.

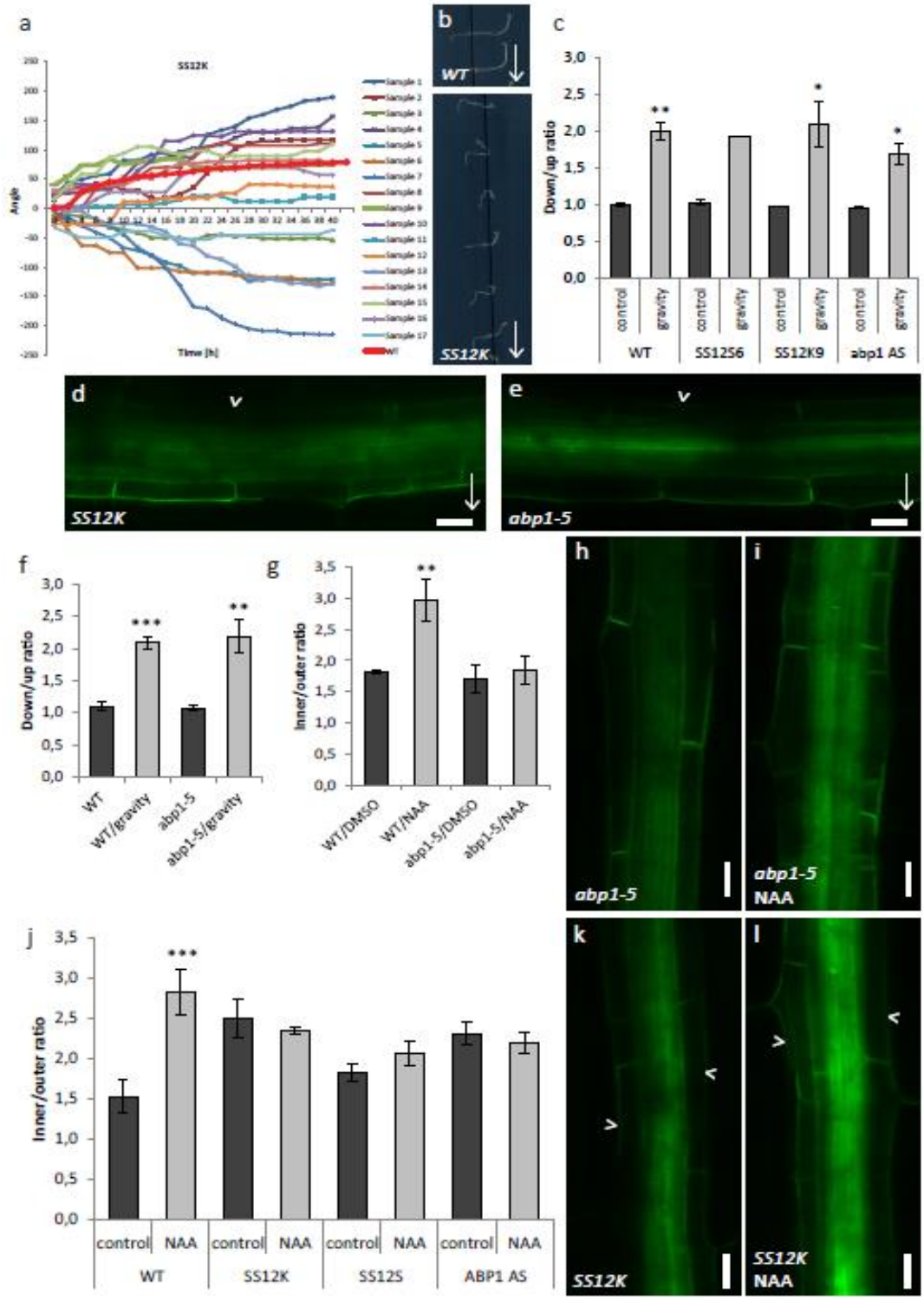

Figure 5. Auxin-induced PIN3 relocation requires ABP1 auxin signaling

(a-f) Gravity response of *abp1* mutants. (a) Bending kinetics in individual hypocotyls of *abp1* knock down (line *SS12K*) mutants show differential bending responses in independent hypocotyls. (b) Examples of the gravitropic response in wild type (WT, top) and *abp1* (*SS12K*, bottom) seedlings. (c) Quantitative evaluation of gravity-dependent PIN3-GFP relocation in endodermal cells of *abp1* knock-down mutants (*SS12S*, *SS12K* and *abp1 AS* lines). Error bars represent standard errors (Student's t test, * $P < 0,05$; ** $P < 0,01$). (d, e) No defects in gravity-induced PIN3-GFP relocation in *SS12K* (d) and in *abp1-5* (e). (f) Quantitative evaluation of gravity-dependent PIN3-GFP relocation in endodermal cells. Error bars represent standard errors (Student's t test, ** $P < 0,01$; *** $P < 0,001$).

(g-l) Resistance of *abp1* mutants to NAA treatment. (h, i) PIN3-GFP localization in *abp1-5* is comparable with (i) or without (h) NAA treatment. (g) Quantitative evaluation of auxin-dependent PIN3-GFP relocation in endodermal

cells. Error bars represent standard errors (Student's t test, ** P < 0,01). (k, l) No PIN3-GFP auxin-induced inner-lateralization in *abp1* knock down line (l; *SS12K*) compared to not treated situation (k). (j) Quantitative evaluation of auxin-dependent PIN3-GFP relocation in endodermal cells. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, *** P < 0,001).
Arrowheads point to PIN3 depletion from outer lateral side of endodermal cells. Scale bars represent 20 µm.

Discussion

Reorienting plant growth according to gravity is one of mechanisms how plants react to the changing environment. Following gravity stimulation, PIN3 relocates in order to modulate the direction of auxin flow toward the bending zone. Increased auxin concentration in the lower side of hypocotyl then stimulates cell elongation and subsequently results in bending of the organ (Friml et al., 2002; Rakusová et al., 2011). However, the steps regarding the fine adjustment of the bending angle are so far not clarified.

Auxin feedback on PIN3 in hypocotyl endodermis to terminate gravity bending response

We were interested to understand how hypocotyls reduce its bending growth to not overbend after gravity stimulation. Correlating the bending gravity response of the hypocotyl to changes in PIN3 localization and DR5 patterns in time identified two phases of gravity response. A bending phase occurs following the gravity stimulation with firstly PIN3 relocating to the bottom side after 2 hours of stimulation, followed by local auxin accumulation after 6 hours. In the meantime, hypocotyl bended fast due to a strong difference in cell elongation between upper and lower sides of the hypocotyl. In a second phase, after 19 hours of gravity stimulation, the hypocotyl reached an almost 90˚bend and slowed down the differential growth (see bending kinetics Figure 1A). We hypothesized that the increased auxin concentration in the bottom side of the hypocotyl between 6 to 32 hours of gravi-stimulation induces PIN3 relocalization to the inner side of endodermal cells, i.e. inner-lateralization (Figure 1E) in order to symmetrically equalize auxin levels between the two sides of the hypocotyl and prevent overbending. The present study reveals the possible role of an auxin feedback on its own accumulation in dark-grown Arabidopsis seedlings to subsequently terminate the gravity-induced bending.

Our results suggest that auxin negatively regulates its own accumulation during the gravity response in hypocotyl. Indeed, when applied exogenously, auxin induces PIN3 inner-lateralization in endodermal cells (Figure 2A-F, Figure S2A and S2B) similar to what is observed in the later stages of gravitropism (Figure 1E). Additionally, similar observations were made in *yucca 1D* seedlings, endogenously overproducing auxin (Figure 2G-2I). NAA

application was further used as the tool to identify the mechanisms of gravity-bending regulation in plants.

To understand whether the auxin effect on PIN3 localization is specific to PIN3 or rather specific for the cell type – in this particular case, endodermal cells, we tested the effect of auxin treatment on different PIN-xFP (*PIN3::PIN3-GFP*, *PIN7::PIN7-GFP*, *SCR::PIN2-GFP* and *SCR::PIN3-YFP*) lines expressed in hypocotyl endodermal cells. All the lines show inner-lateralization after auxin application (Figure S3). Thus, the decision about lateral polarity induced by auxin appears to be cell type-specific rather than sequence specific.

Intracellular regulation of PIN3 polarity changes

To further characterize auxin-mediated changes of PIN3 localization we used mutants or available chemical inhibitors to interfere with auxin signaling, PIN polar sorting, endocytosis and recycling. Taken together, PIN3 response to auxin is regulated on several levels and includes clathrin-mediated endocytosis, GNOM ARF GEF-dependent protein cycling and involves PID-dependent phosphorylation as regulatory clue for PIN3 polarity.

Separate measurements of PIN3-GFP fluorescence intensity in the outer and inner lateral side suggest that auxin treatment induced rather a depletion of PIN3-GFP from the outer side of endodermal cells than relocation to the inner-side since the signal in the outer lateral side decreased, but did not increase in the inner-lateral side (Figure S1 and Figure S5K). Hypothesis was supported by using inhibitors of protein degradation (Figure 3I-3L, Figure S3). We suggest the conservative role of stabilization and subsequently degradation of PINs to deplete PIN3 from outer PM as shown for PIN2 in the root tip during gravity response (Abas et al., 2006; Baster et al., 2013).

As PID had previously been implicated in the regulation of PIN3 polarity in previous studies (Ding et al., 2011; Rakusová et al., 2011), we examined whether changes in phosphorylation status is causative the auxin effect on PIN3 polarity. Indeed, gain-of-function PID hypocotyls were resistant to the external auxin application for PIN3 inner-lateralization (Figure 4). In contrast, when the PID-dependent phosphorylation pathway was altered, seedling hypocotyls displayed hyper-gravitropic response (Rakusová et al., 2011) and already inner-lateralized PIN3 regardless of auxin application (Figure 4). The opposite effects of the *wag1 wag2 pid* points to a more complex role of PID and related kinases in gravitropism. These observations suggest the possible role in a PID-dependent feed-back for the regulation of

hypocotyl bending after gravity stimulation using phosphorylation as a signal to maintain PIN3 at the PM.

Notably, auxin effect on PIN3 polarity appears to be mediated primarily by the ABP1 auxin receptor signaling but does not strictly require function of the TIR1/AFB nuclear auxin receptors. However, given that the both ABP1 and TIR1/AFB pathways are extensively mutually regulated (Tromas et al., 2013), dissecting their contributions separately is difficult.

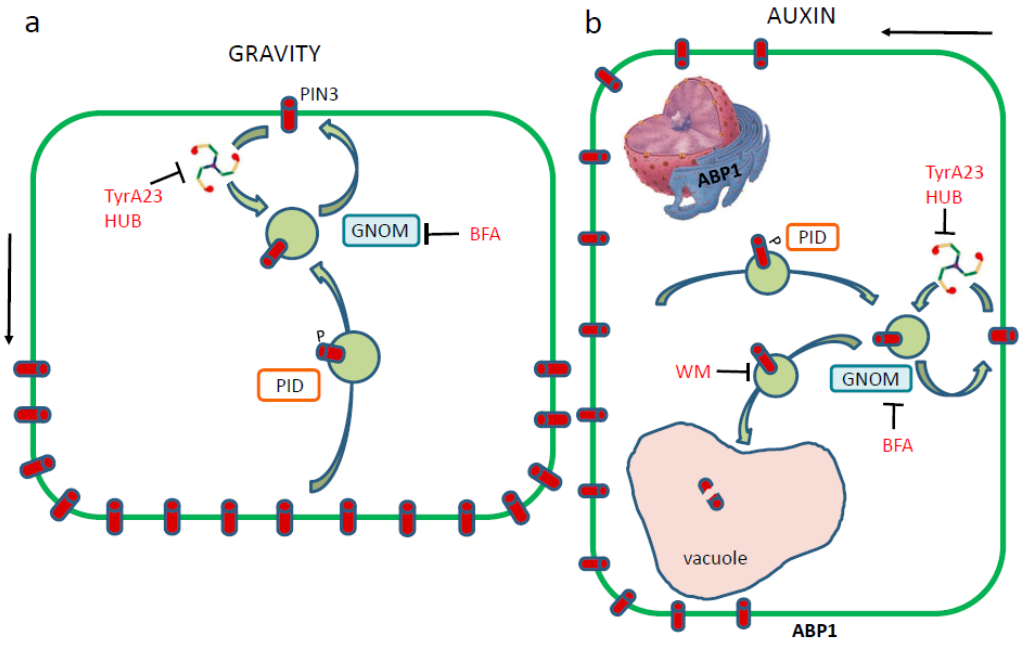

Figure 6. Model of PIN3 polarity regulation in endodermal cells during hypocotyl gravitropism.

(a) Gravity-induced PIN3 relocation relies on clathrin-dependent endocytosis, GNOM-regulated cycling and PINOID-mediated phosphorylation. Arrow points the gravity direction.

(b) Auxin-induced PIN3 inner-lateralization is clathrin- and GNOM-dependent, requires PINOID-mediated phosphorylation and seems to rely also on PIN3 degradation. The process is mediated by ABP1 auxin receptor that can be found in the ER or in the apoplast. Arrow points the outer-to-inner direction.

WM – wortmannin; ABP1 – Auxin Binding Protein 1; PID – PINOID; TyrA23 – tyrphostin A23; BFA – brefeldinA; P - phosphorylation.

Our observations revealed two temporary and mechanistically separated events of PIN3 polarization in endodermal cells during the gravitropic response – the earlier gravity-induced relocation to the inner lateral side at the upper side of the shoot and later auxin-induced PIN3 inner-lateralization at the lower side (Figure 6). These subsequent polarizations are consistent with initial auxin flow towards the lower side and auxin accumulation there followed by equalization of auxin levels between lower and upper side and onset of symmetric shoot growth. Thus our results provide a previously unexplained concept of how gravitropic and possibly also phototropic responses are terminated. Our results suggest that it is ABP1-mediated auxin feed-

back on PIN3 polarization leading to reduction of auxin asymmetry and the termination of tropic bending.

Experimental Procedures

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia was used in the study as the plant material. Published transgenic and mutant lines were used: *PIN3::PIN3-GFP* (Žádníková et al., 2010), *PIN7::PIN7-GFP* (Blilou et al., 2005), *SCR::PIN3-YFP* (Rakusová et al., 2011), *SCR::PIN2-GFP* (Xu et al. 2006), *DR5rev::GFP (Friml et al., 2003)*, *GNOM*^{M696L} (Geldner et al., 2003), *wag1 wag2 pid-14/+* (Dhonukshe et al., 2010), *35S::PID-21* (Benjamins et al., 2001), *35S::ABP1* (Rakusova and Wabnik, 2014), *tir1 afb2 afb3* (Dharmasiri et al., 2005), *pINTAM>>RFP-HUB1* (before the experiment, lines were induced overnight with 2 µM tamoxifen.; (Robert et al., 2010)), *abp1* knockdown lines (*SS12S*, *SS12K* and *AS*; (Braun et al., 2008), lines were induced after germination with evaporating 8% ethanol),, *yucca 1D* (Zhao et al., 2001). Mutants with *PIN3::PIN3-GFP* were generated by crossing.

Plants were sown on ½ MS plates with 1% sucrose, 8 g/L agar (pH 5,8). Seeds were vernalized for 2 days at 4°C, exposed to light for 5-6 hours at 18°C, and cultivated in the dark at 18°C. For gravitropic stimulations, plates with 4-day-old etiolated seedlings were rotated 90°. Seedlings were either scanned for measurements (with Image J), analyzed by real-time phenotype analysis or imaged by confocal microscopy. Each experiment was conducted at least in triplicate. For confocal microscopy, an LSM 700 confocal scanning microscope (Zeiss, http://www.zeiss.com) was used.

Real-time analysis

Gravity response of seedlings was recorded at 1 or 2-hour intervals for 1 or 2 days at 18°C with an infrared light source (880 nm LED; Velleman, Belgium) by a spectrum-enhanced camera (EOS035 Canon Rebel Xti, 400DH) with built-in clear wideband-multicoated filter and standard accessories (Canon) and operated by the EOS utility software. Angles of hypocotyls were measured by ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij). Minimum 20 seedlings with synchronized germination start were processed.

Quantitative analysis of DR5 and PIN3 relocation

The rate of the fluorescence of *DR5rev::GFP* was compared between the lower and upper side of the hypocotyl in the responsive part. For quantification of the gravity-induced PIN3-GFP relocation the rate of PIN3-GFP fluorescence intensity was compared between the external PM sides of endodermal cells. Fluorescence intensity was measured by ImageJ using simple line along the plasma membrane. This method awoids interrupting signal from the stele. The PIN3 relocation is well visible in the upper endodermal cells, since the lower cell signal is influenced with PIN3-GFP signal in stele. For the auxin-induced PIN3-GFP relocation the mean fluorescence intensity of PIN3-GFP signal at the inner lateral and the outer lateral membrane of endodermal cells were measured using ImageJ software. Three replicates of at least 10 seedlings with a synchronized germination start were processed. The presented value is the mean of the averages.

Pharmacological treatments

Wild type seedlings were germinated and grown on vertical ½ MS with sucrose agar plates at 18 or 21℃ for 4 days. Treatments in the dark were performed by transfer and incubation of 4-day-old etiolated seedlings on solid medium supplemented with NAA (10 µM if not stated otherwise, Duchefa), IAA (10 µM if not mention otherwise, Duchefa), BFA (50 µM, Sigma), MG132 (25 µM, Calbiochem), WM (30 µM, Sigma), CHX (50 µM, Sigma), Naphthalene (25 µM, Fluka), PEO-IAA (25 µM), 5F-IAA (25 µM, OlChemIm (Olomouc, Czech Republic)), Auxinole (25 µM, (Hayashi et al., 2012)), Tyrphostyn23 and Tyrphostyn51 (30 µM, both Sigma). All co-treatments with NAA were done after 1h of pre-treatment with the drug followed by 4h co-treatment with NAA or 6 hours gravity stimulation. Control treatments contained an equivalent amount of solvent (DMSO, Sigma). For all comparisons, at least three independent experiments were carried out, giving the same significant results.

Supplemental data

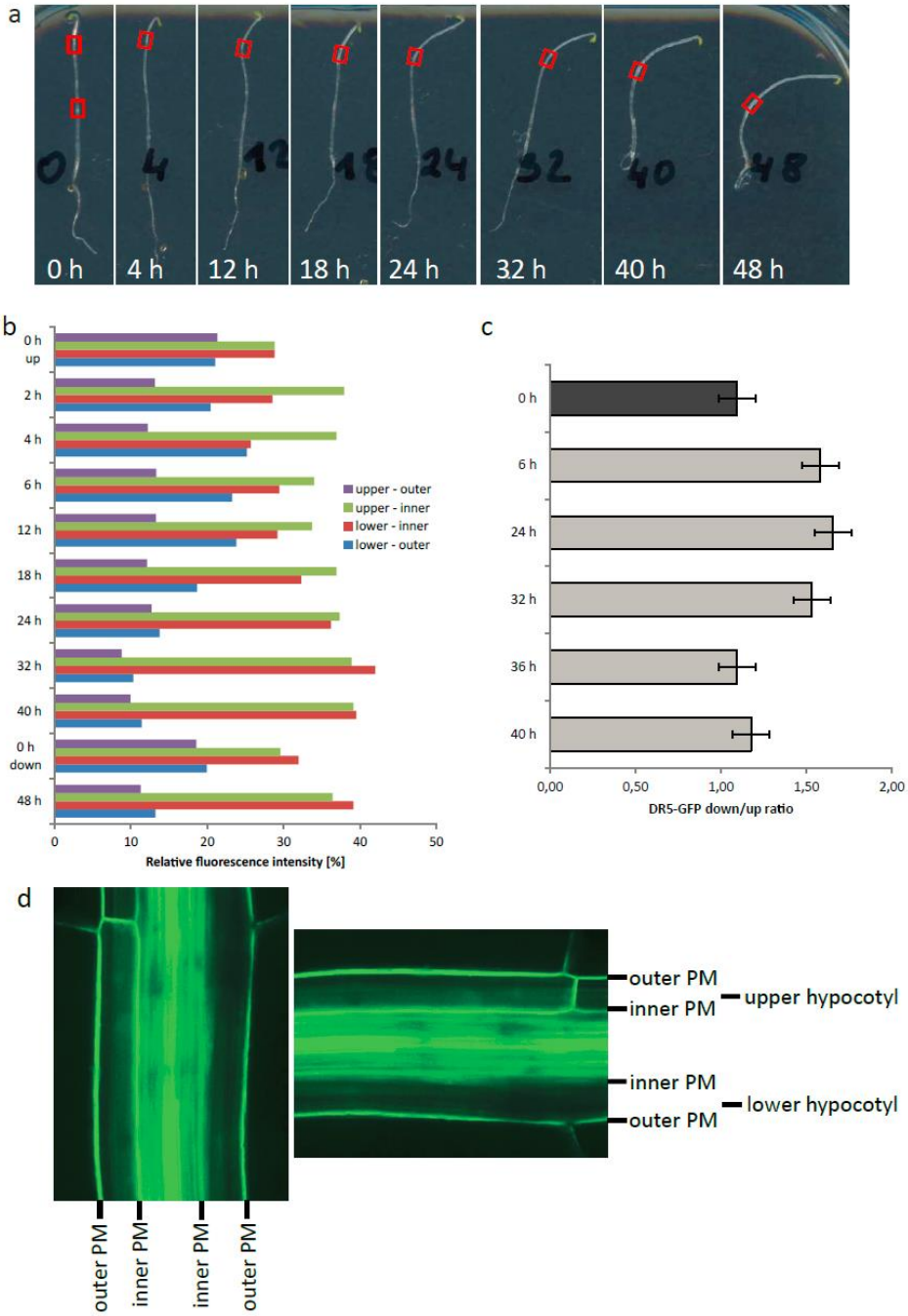

Figure S1. Evaluation of PIN3 polarity changes and DR5 accumulation during the gravity response in hypocotyls

(a) Approximate position at the hypocotyl where the most pronounce bending angle was present. The place was also identical with the position where gravity-induced PIN3 polarization was observed. Control (0h) was measured in 2 different positions – up and down on the hypocotyl and compared based on different position of angle in stimulated hypocotyls.

(b) Quantification of gravity-mediated PIN3 polarization during different time points of gravity stimulation. PIN3-GFP fluorescence intensity was measured independently in outer and inner endodermis cell sides of upper and lower side of hypocotyl. Graph represents percentage of PIN3-GFP fluorescence intensity for independent endodermis cell side in independent time points.

(c) Asymmetric distribution of the *DR5rev::GFP* auxin response reporter in wild type hypocotyl during different time points after gravity stimulation.

(d) Scheme of PIN3 plasma membrane (PM) localization in control and gravity-stimulated hypocotyl endodermal cells.

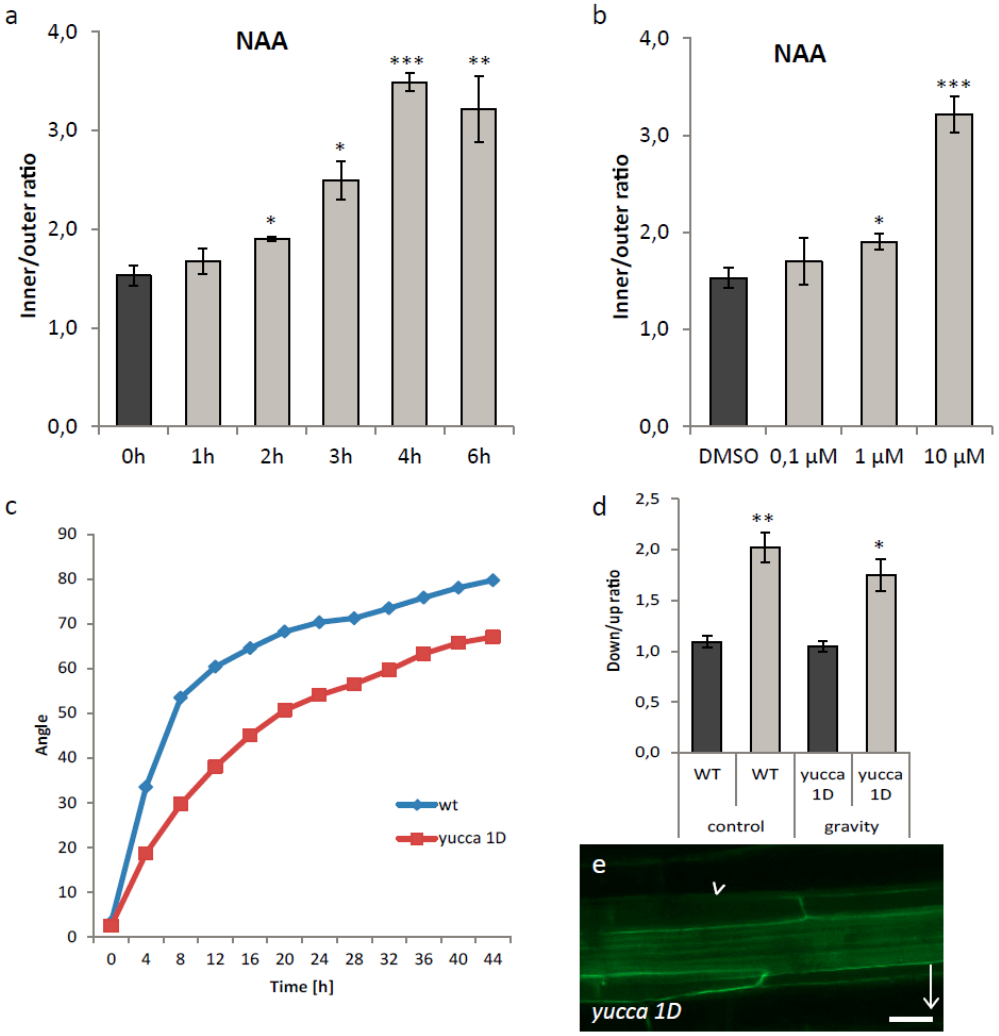

Figure S2. Auxin-dependent changes of PIN3 polarity

(a, b) Time (a) and concentration (b) dependence of auxin effect using 10 µM NAA for different time points or 4h treatments with different NAA concentrations, respectively. Graphs show mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, * P < 0,05; ** P < 0,01; *** P < 0,001).

(c) Kinetic curves of hypocotyl gravitropic response in *yucca1D* mutant (red) and in wild type (blue).

(d, e) Ratio of PIN3-GFP signal intensities between outer lower and upper endodermal cells membrane (down/up ration) in *yucca1D* mutant and wild type in control conditions and after gravity stimuli (d). Confocal picture of PIN3-GFP in *yucca1D* hypocotyl after gravity stimuli (e). Arrowheads point PIN3-GFP depletion from outer lateral side of endodermal cells. Arrow marks direction of gravity. Scale bar represents 20 µm.

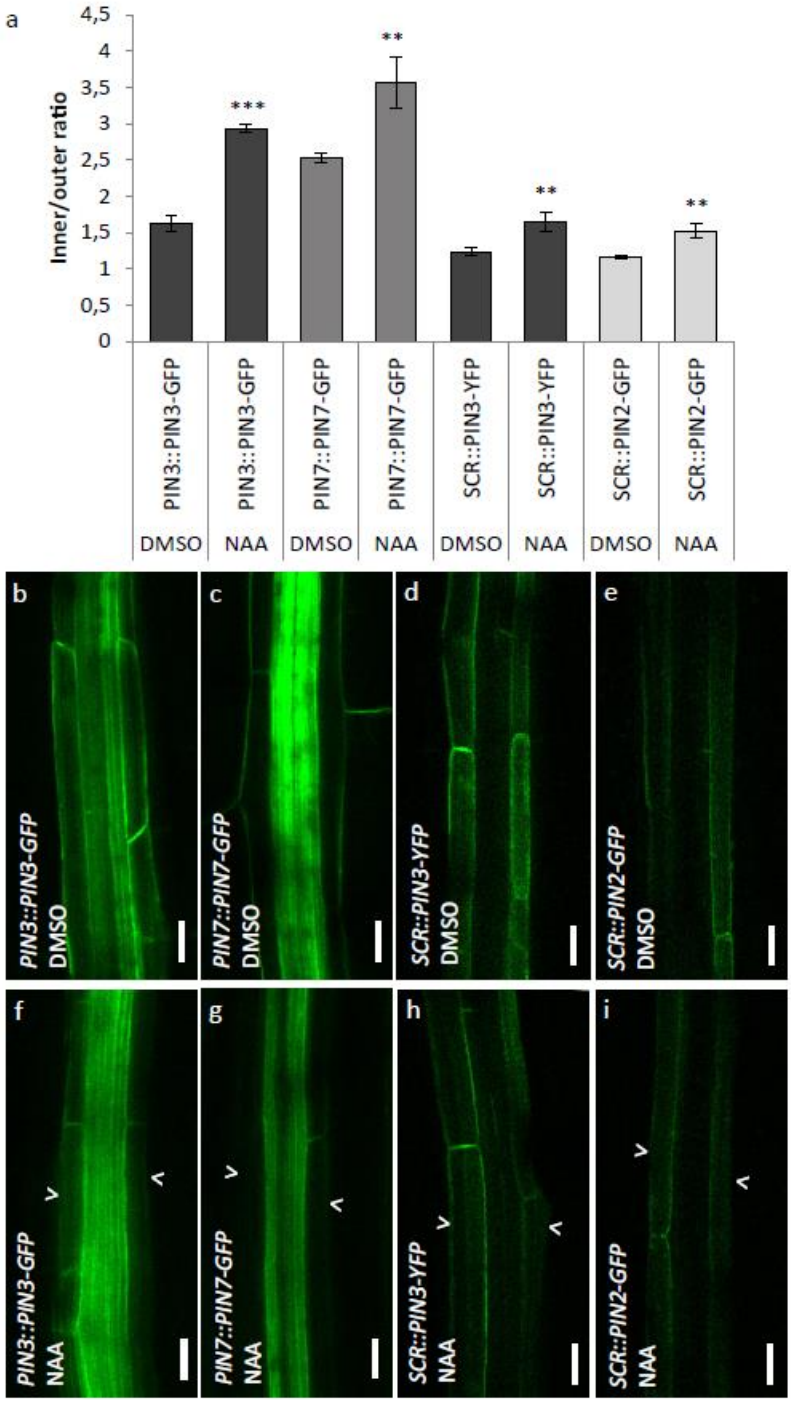

Figure S3. Cell-specific auxin effect on PIN inner-lateralization

(a) Quantitative evaluation of auxin-dependent PIN3-GFP, PIN7-GFP, PIN3-YFP and PIN2-GFP relocation in endodermis cells. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, ** $P < 0,01$; *** $P < 0,001$).

(b-i) Effect of auxin treatment on PIN3-GFP (f), PIN7-GFP (g), PIN3-YFP (h) and PIN2-GFP (i) localization in hypocotyl endodermis compared to untreated controls (b-e). *SCR::PIN3-YFP* (d, h) was used here as control for *SCR::PIN2-GFP* (e, i) where the expression is specifically in endodermal cells. Arrowheads point to PIN3-GFP depletion from outer lateral side of endodermal cells. Scale bars represent 20 µm.

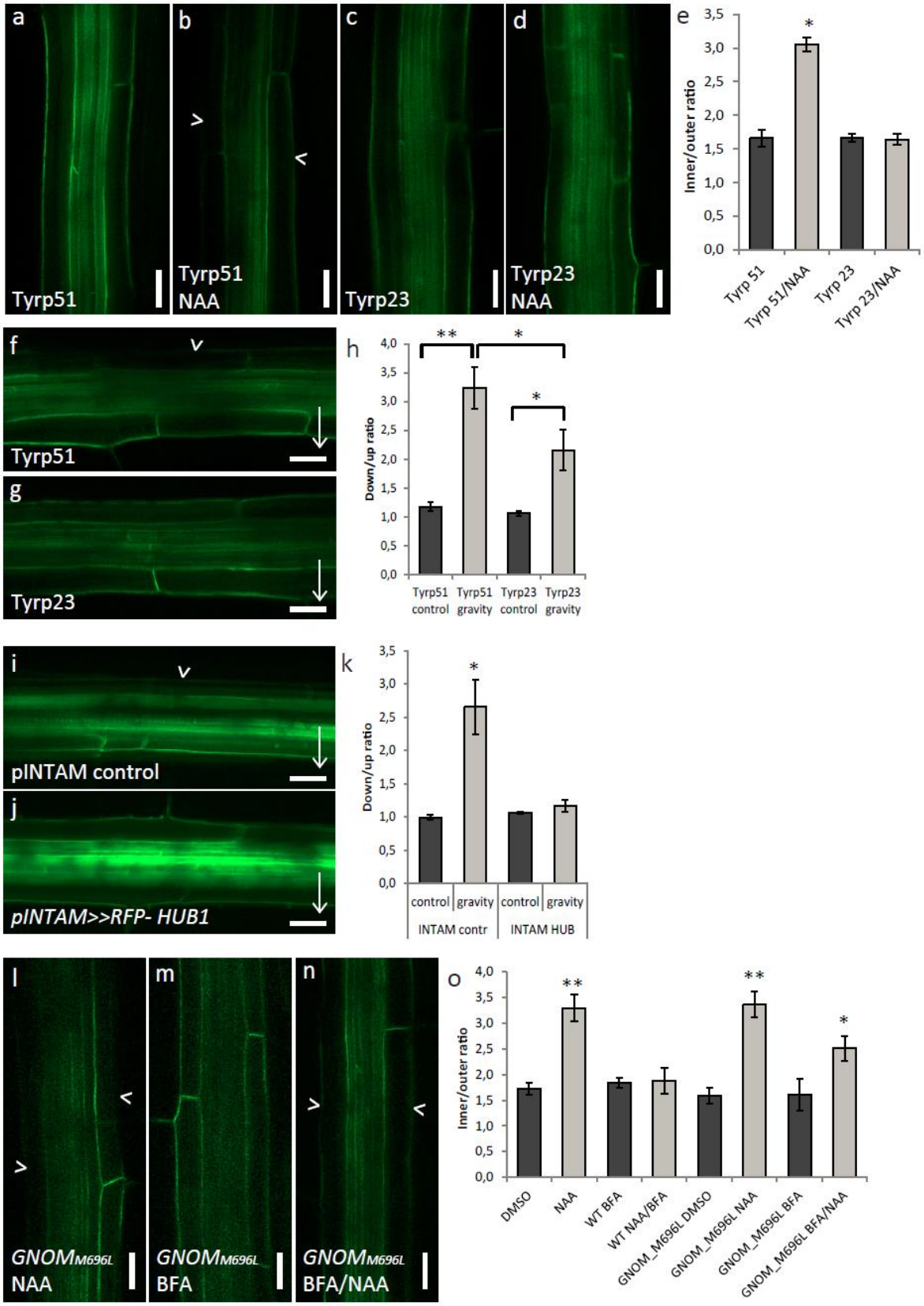

Figure S4. Clathrin- and GNOM-mediated endocytosis plays a role during gravity- and auxin-mediated PIN3 polarity changes

(a-d) Inhibition of clathrin-mediated endocytosis did not affect steady state PIN3 apolar localization, shown by treatment with tyrphostin23 (Tyrp23) (c) compared to the inactive analog tyrphostin51 (Tyrp51), as control (a). Auxin-induced PIN3-GFP inner-lateralization was inhibited by Tyrp 23 (d), but not by Tyrp51 (b).

(e) Quantitative evaluation of auxin-dependent PIN3-GFP relocation in endodermis cells. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, * $P < 0,05$).
(f-h) Tyrp 23 (g) effects on PIN3-GFP gravity-induced polarization in hypocotyl endodermal cell compared to its inactive analog Tyrp51 (f). (h) Graph displaying the ratio of PIN3-GFP fluorescence intensity at the outer side of endodermis cells at the lower versus upper hypocotyl side after gravity stimulation on Tyrp23 and Tyrp51 as negative control. Error bars are SEs (Student's t-test, * $P < 0,05$; ** $P < 0,01$).
(i-k) Induced *pINTAM>>RFP-HUB1* showed inhibition of PIN3-GFP polarization after gravity stimulation compared to the control (i). Before the experiment, lines were induced overnight with 2 µM tamoxifen. (k) Ratio of PIN3-GFP fluorescence intensity at the outer side of endodermis cells at the lower versus upper hypocotyl side after gravity stimulation. Error bars represent standard errors (Student's t test, * $P < 0,05$). Arrowheads point PIN3-GFP depletion from outer lateral side of endodermal cells.
(l-o) Brefeldin A (BFA) effects auxin-induced PIN3-GFP relocation in hypocotyl endodermal cells. NAA (l), BFA (m), and NAA/BFA (n) treatments in the BFA-resistant $GNOM^{M696L}$ line showed no interference with PIN3-GFP inner-lateralization. Ratio of PIN3-GFP fluorescence intensity at the outer and inner side of endodermis cells at hypocotyl after BFA treatment or BFA/NAA co-treatment (o). Error bars are SEs (Student's t-test, * $P < 0,05$; ** $P < 0,01$). Arrowheads point PIN3-GFP depletion from outer lateral side of endodermal cells. Scale bars represent 20 µm.

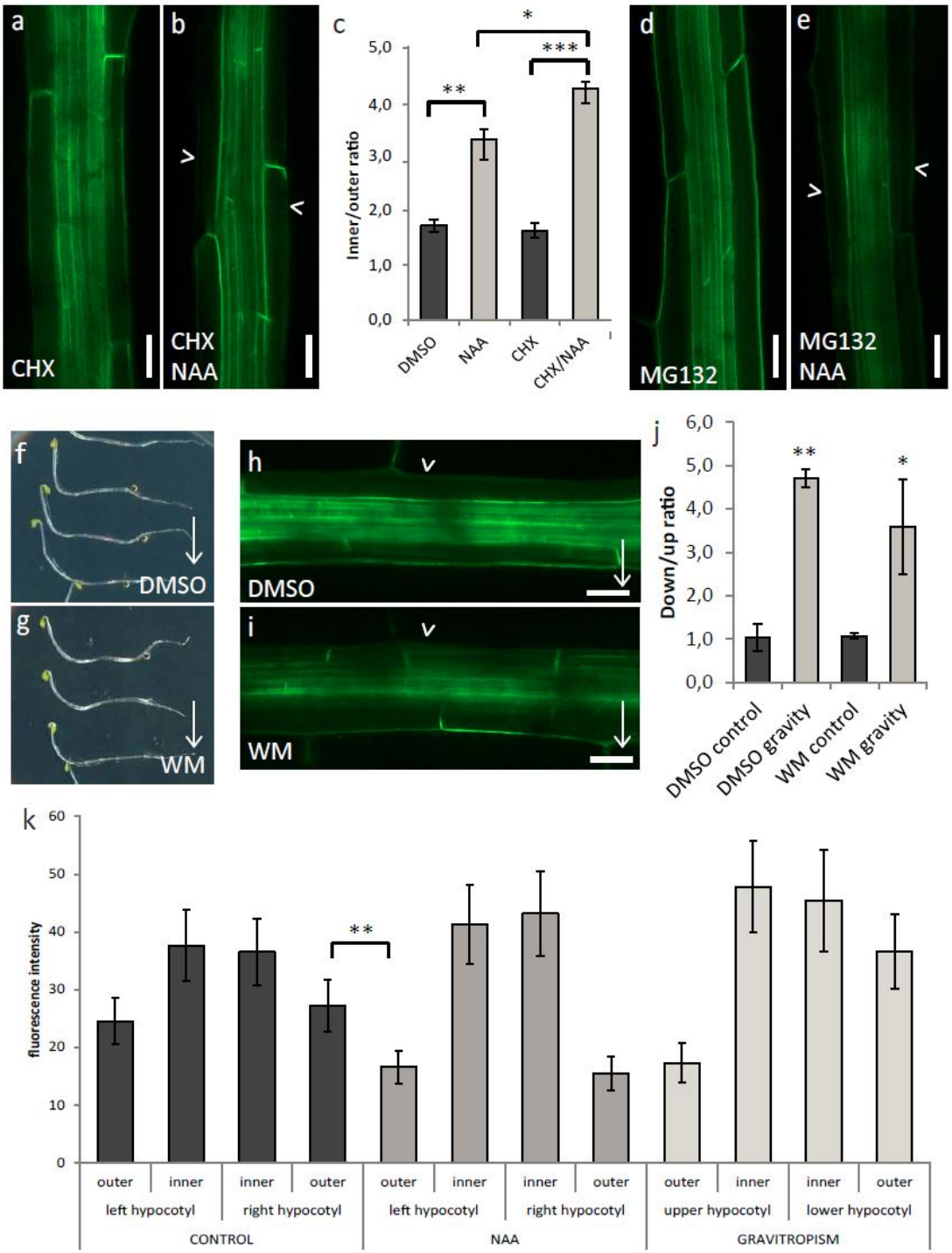

Figure S5. Role of protein synthesis and degradation during gravity- and auxin-mediated PIN3 polarity changes

(a-c) No altered PIN3-GFP localization in the hypocotyl endodermis was observed when treated on plates supplemented with CHX (a). Co-treatment CHX with NAA showed higher sensitivity in auxin-mediated PIN3-GFP inner-lateralization in endodermal cells (b). (c) Quantitative evaluation of auxin-dependent PIN3-GFP relocation in endodermal cells. Error bars are SE (Student's t test: * $P < 0,05$; ** $P < 0,01$; ***$P < 0,001$).

(d, e) No altered PIN3-GFP localization in the hypocotyl endodermis was observed when treated on plates supplemented with MG132 (d). MG132/NAA co-treatment showed only a slight reduction of PIN3-GFP inner-lateralization after NAA treatment (e). Arrowheads point PIN3 depletion from outer lateral side of endodermal cells. For quantification, see Figure 3I.

(f-j) No inhibition of hypocotyl gravity response was observed when treated with wortmanin (WM) (g) compared to control (f). Gravity-induced PIN3-GFP polarization in hypocotyls after wortmanin pretreatment followed with gravity stimulation (i). Non-treated control (h). Ratio of PIN3-GFP fluorescence intensities at the outer side of

endodermis cells at the lower versus upper hypocotyl side (j). Error bars are SE (Student's t test: * P<0,05; ** P<0,01).
(k) Relative PIN3-GFP signal at the outer and inner lateral cell sides of the left hypocotyl side or inner and outer lateral cell sides of the right hypocotyl side. The measurements were performed in the same focal plane. Units are arbitrary fluorescence units (AU). Independently measured PIN3-GFP fluorescence intensity shows depletion of PIN3-GFP from the outer side of endodermal cells after NAA treatment. Graph shows mean signal intensity separately for independent endodermal cell sides in hypocotyl. Error bars represent standard errors (Student's t test, ** P < 0,01). Scale bars represent 20 µm.

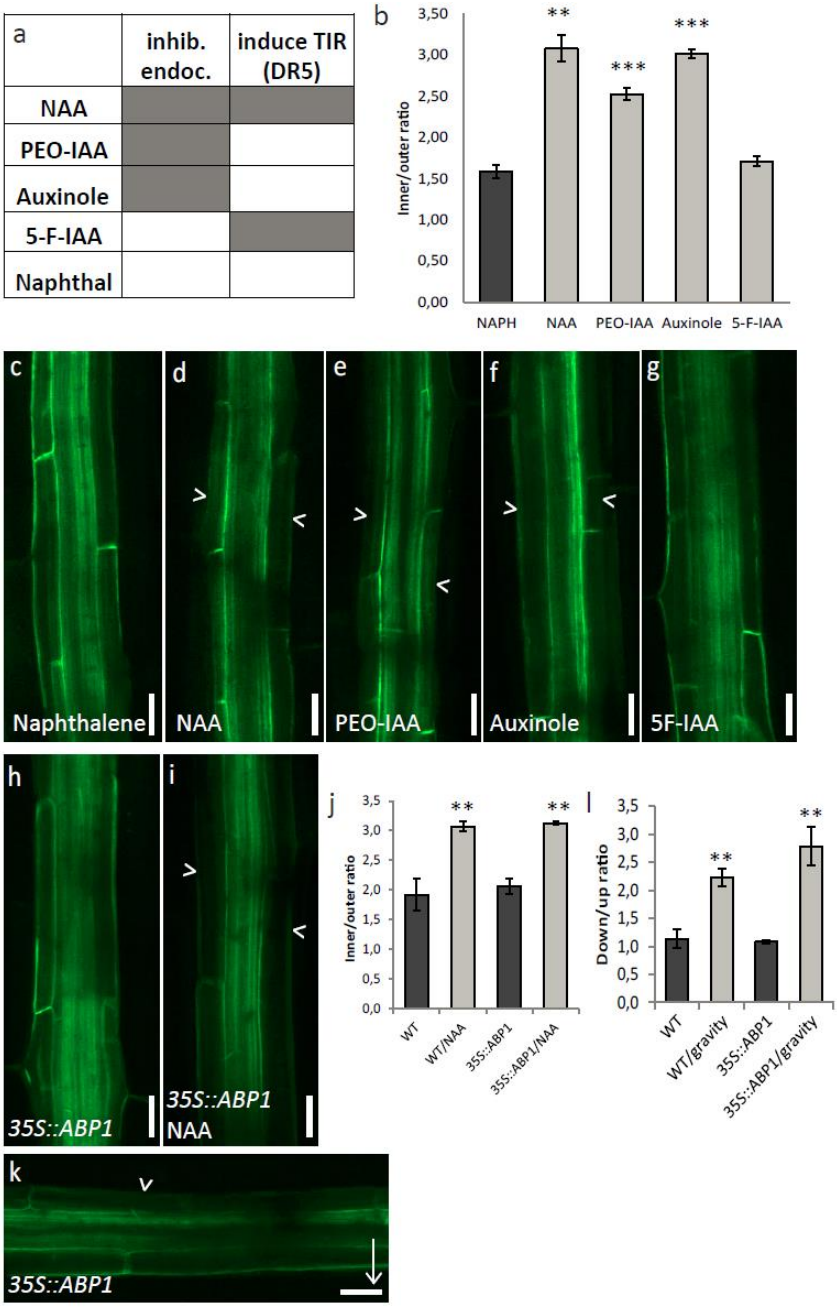

Figure S6. ABP1, but not TIR1, dependent auxin-induced PIN3 relocation
(a) Table shows differential effects of auxin analogs for endocytosis inhibition and *DR5* activation. PEO-IAA and auxinole inhibited ABP1-dependent endocytosis but did not induce TIR1 dependent pathway. 5-F-IAA did not inhibit endocytosis but induce TIR1 signaling pathway (according (Robert et al., 2010)). Naphthalene was used as negative control.
(b) Quantitative evaluation of auxin analogs-dependent PIN3-GFP relocation in endodermis cells. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, ** P < 0,01; *** P < 0,001).

(c-g) Effect of auxin analogs on PIN3 localization. NAA, PEO-IAA and auxinole induced PIN3-GFP inner-lateralization (d, e, f) compared to control (c). 5F-IAA did not influence PIN3-GFP localization (g). All the compounds were used in 25 µM concentration for 4h. Arrowheads point to PIN3-GFP depletion from outer PM of endodermal cells.
(h-j) Overexpression of ABP1 (*35S::ABP1*) did not influence nor PIN3-GFP apolar localization (h) neither auxin-induced PIN3-GFP inner-lateralization (i). (j) Quantitative evaluation of auxin-dependent PIN3-GFP relocation in endodermal cells. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, ** P < 0,01).
(l, k) Gravity-induced PIN3-GFP polarization in *35S::ABP1* hypocotyls (k). (l) Ratio of PIN3-GFP fluorescence intensities at the outer side of endodermis cells at the lower versus upper hypocotyl side after gravistimulation. Error bars represent standard errors (Student's t test, ** P < 0,01). Scale bars represent 20 µm.

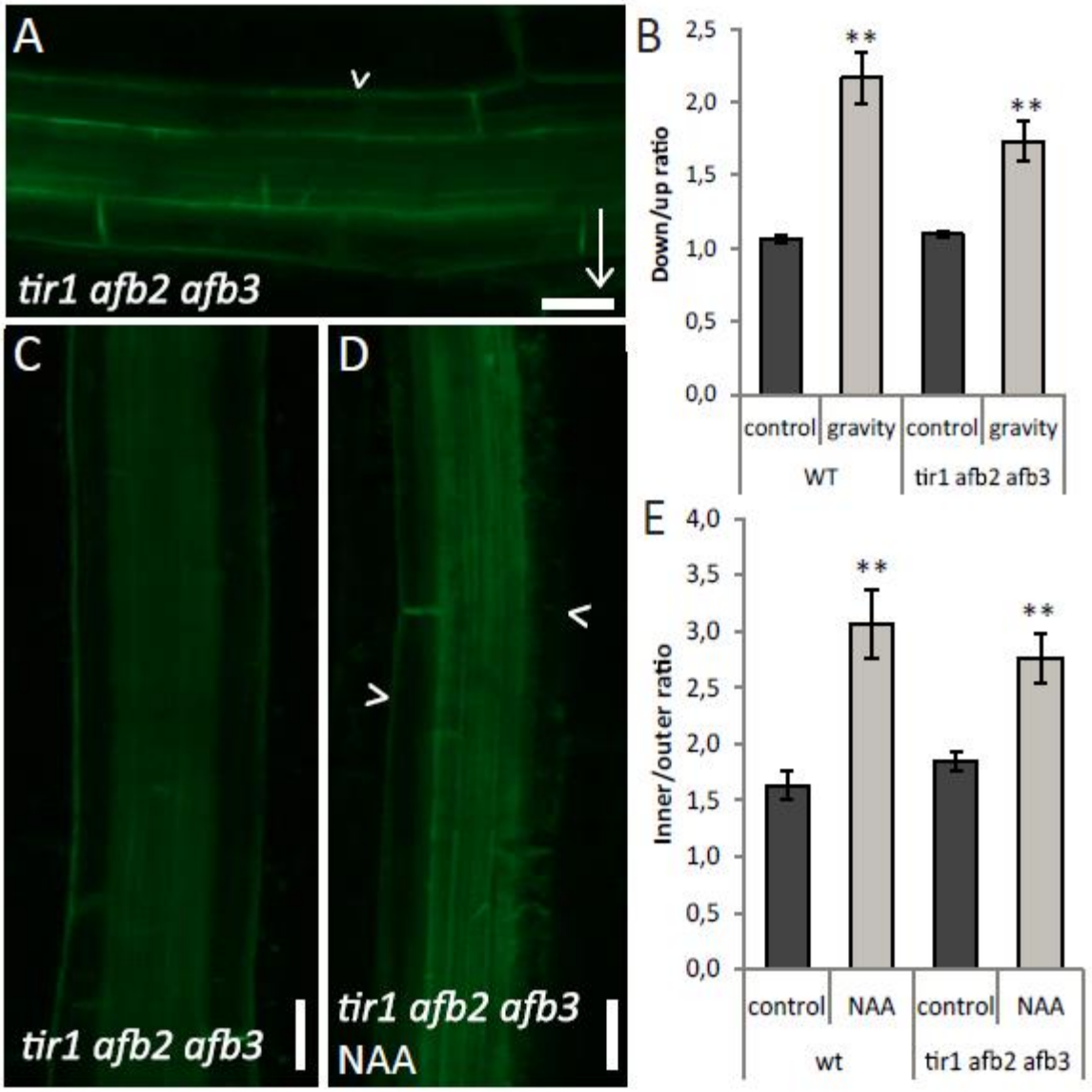

Figure S7. TIR1-dependent auxin-induced PIN3 relocation
(a-c) Gravity-induced PIN3-GFP relocation in *tir1 afb2 afb3* triple mutant showed no difference with wild type (b). (b) Quantitative evaluations of gravity-dependent PIN3-GFP relocation in endodermis cells. Arrows mark gravity direction.
(c-e) Auxin-induced PIN3-GFP inner-lateralization in *tir1 afb2 afb3* triple mutant (d) compared to the untreated mutant (c). (e) Quantitative evaluation of PIN3-GFP inner-lateralization in endodermis cells. Graph shows mean ratio of inner to outer signal intensity. Error bars represent standard errors (Student's t test, ** P < 0,01). Scale bars represent 20 µm.

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Chapter 4.

Identification of new PIN3 polarity regulators in Arabidopsis gravity-stimulated hypocotyls

HR and JF initiated the project and designed the experiments, HR carried out all the experiments.

Identification of new PIN3 polarity regulators in *Arabidopsis* gravity-stimulated hypocotyls

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Abstract

Tropism is a mechanism by which plants adapt to environmental changes, such as light - phototropism and gravity - gravitropism. Gravitropism is a growth reaction that orients the plant's development parallel to the gravitational field of the earth. Roots grow with the gravity vector, whereas shoots grow against it. Gravity-induced redistribution of the phytohormone auxin mediates tropic responses both in roots and in shoots. In the two instances, the PIN-FORMED3 (PIN3) auxin transporter relocalizes in response to gravistimuli to redirect the auxin flow and to achieve an asymmetric auxin distribution. Thus, the control of the PIN3 subcellular localization and the subsequent PIN3-dependent auxin transport are crucial processes for tropic bending. We used a forward genetic screen to find still undiscovered cellular components involved in the regulation of the PIN3 repolarization after light- and/or gravity stimulation. The screen was based on the gravitropic hypocotyl growth of the transgenic *PIN3::PIN3-GFP* line. We searched for mutants with an altered PIN3 relocation, leading to easy-to-score morphological outputs that are visible during the hypocotyl bending. The screen had been designed based on the hypothesis that the EMS-induced mutations in the putative PIN3 repolarization regulators will lead to a defect in gravity-mediated bending. This forward genetic screen would identify new PIN3 polarity components and provide us with more insight into the mechanism of PIN3 cell polarity establishment, maintenance, and dynamic regulation in plants. Based on the screen, 28 novel mutant alleles with defective bending or overbending responses were recovered. Nine selected candidates were sequenced by means of whole-genome sequencing and two were characterized in more detail.

Introduction

Plants exhibit developmental plasticity to adjust to changing environmental conditions. Phototropism and gravitropism are two examples of how plants flexibly adapt their growth in response to light and gravity. The key player in this process is the phytohormone auxin and its asymmetric distribution in response to light or gravity stimuli (Friml et al, 2002). Auxin accumulates at the lower side of roots and hypocotyls/stems during gravity stimulation or at the shaded side of hypocotyls in case of unilateral light stimulation and induces cell elongation in the hypocotyl and inhibits cell elongation in root. Asymmetric cell elongation results in organ bending.

The regulation of the cell-to-cell auxin flow is based on the polar localization of the PIN-FORMED (PIN) auxin efflux carriers (Wiśniewska et al, 2006). PIN proteins are plasma membrane (PM) auxin transporters of which the intracellular polarization controls auxin transport and accumulation during various developmental processes and during tropic responses (Kleine-Vehn and Friml, 2008). Based on their expression pattern, localization, and polarity regulation after tropic stimuli, PIN3 and PIN7 are the main candidates that regulate tropic-dependent auxin gradients. PIN3 is expressed in columella cells in roots and in stele and endodermal cells in hypocotyls. Based on gravity or light stimuli, PIN3 relocates to the lower or shaded endodermal cell side and controls the auxin stream to the lower or shaded side of roots or hypocotyls (Ding et al, 2011; Friml et al, 2002; Kleine-Vehn et al, 2010; Rakusová et al, 2011). Thus, the PIN3 subcellular regulation, upstream of the auxin gradient formation and tropic-induced organ bending, is crucial in studies focusing on auxin-dependent tropic responses in plants. To avoid hypocotyl overbending during the gravity bending response auxin feeds back on the PM localization of PIN3 in endodermal cells at the lower side of hypocotyls, resulting in a reduction of its own accumulation (Chapter 3). After the establishment of increased auxin concentrations in these cells, PIN3 at the outer cell side is specifically targeted for lytic degradation and remains at the inner cell side. By restricting PIN3 on the inner cell side, the auxin gradient flattens in the lower epidermis and cells stop to elongate, resulting in bending termination (see Chapter 3). We hypothesized that defects in auxin-mediated PIN3 inner-lateralization would cause an overbending response of hypocotyls.

Intracellular PIN3 polarity regulation is based on actin-dependent constitutive protein cycling via clathrin- and ARF-GEF GNOM-mediated endocytosis. Relocation depends on inhibition of the kinase activities of PINOID (PID) and related WAG1 and WAG2 serine/threonine protein kinases. Previously, we have shown that high levels of PID kinase

expression can inhibit the polar PIN3 recruitment (Chapters 2 and 3) (Ding et al, 2011; Rakusová et al, 2011).

Here we describe a forward genetic screen as an approach to characterize the so-far unknown PIN3 polarity regulators. By using the tropic response of etiolated hypocotyls as a model, we selected specifically the potential regulators for the PIN3 polarization during tropic responses. The selected candidates were divided into two groups. One group comprises mutants without response to light and gravity. The other group includes mutants with hypocotyl overbending phenotypes. We assumed that candidates from the first group may contain mutations in pathways regulating the PIN3-mediated tropic responses. In contrast, candidates from the second group may carry mutations in genes involved in auxin-induced PIN3 polarity changes after the initial bending response. Detailed characterization of mutant candidate phenotypes suggest that some mutants may represent molecular components that control PIN3 polarity changes and subsequently affect auxin-mediated responses in hypocotyls.

Results

A forward genetic screen to identify mutant candidates defective in PIN3 polarity regulation

To identify new molecular components of the mechanism that relocates PIN3 during gravitropic and phototropic responses, we developed a forward genetic screen that would allow us to isolate mutations linking PIN3 localization and gravitropic and phototropic signaling pathways. The screen related to gravitropism and phototropism responses was done in parallel. Mutants with defects in both phototropism and gravitropism responses were preferentially selected, because defective responses to both light and gravity stimuli suggested that the mutated genes did not alter any of the stimuli perception, but rather were affected in common downstream signaling pathways leading to the hypocotyl bending. We also preferentially selected mutants with a normal hypocotyl growth (elongation), indicating that the observed phenotypes with altered bending responses were not caused by a general defective cell elongation. We divided the mutants into two categories. One group contained mutants with defective bending or unresponsive to gravity vector changes. We assumes that these mutants would have specific defects in gravity-induced PIN3 polarity changes. The other category included mutants showing an overbending response to gravity and light. Mutants from this group were predicted to lack the auxin feedback on the PIN3 inner-lateralization at later stages of hypocotyl bending.

An ethyl methyl sulfonate (EMS)-mutagenized *PIN3::PIN3-GFP* population was established and screened for mutants affected in phototropic and gravitropic responses and on the polar PIN3 localization. This strategy enabled us to screen macroscopically for mutants based on phenotypes after tropic stimulations. We designed and optimized the primary screen of ~ 66 800 M2 (in 100 M2 pools representing 2671 M1) 4-day-old etiolated seedlings based on the resistance or hypersensitivity to gravity changes after 24 hours of stimulation. A secondary screen on 127 selected M2 candidates in the M3 generation was done by testing both tropic responses (gravi- and phototropism), confirming the heritable and stable defects in the gravitropic bending response. These selected candidates were analyzed for altered phototropism response as well. Moreover, the PIN3-GFP relocation in the endodermal cells after gravistimuli was checked in the 28 candidates with the strongest phenotypes by means of confocal microscopy 6 hours after gravistimulation.

In addition, another important analyzed phenotype related to the PIN3 contribution in hypocotyl growth was the closure and aperture of the apical hook structure (Žádníková et al., 2010). All the phenotypes described above were compared to select the most interesting candidates (Figure 1 and 2; Table 1). After the secondary screen, the phenotype in selected candidates was confirmed, including the PIN3 relocation defects after gravity, light stimulation, and NAA treatment as well as the root gravity response and apical hook formation.

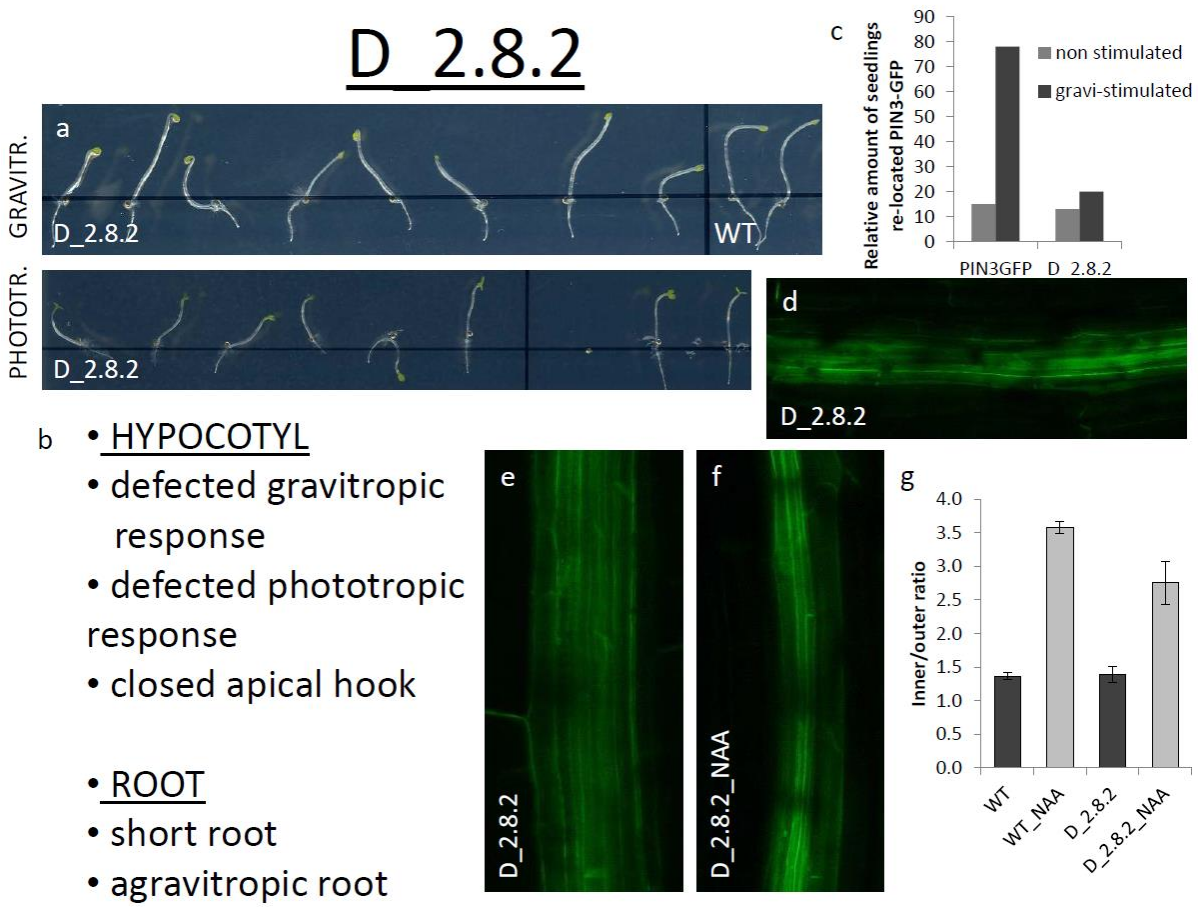


Figure 1. Mutant with agravitropic phenotype from forward genetic screen.
(a) Mutant D_2.8.2 with defected hypocotyl responses to gravity or light stimuli. (b) List of mutant phenotypes. The growth of hypocotyls and roots is normal under light conditions and the apical hook is closed in the dark. (c-e) The PIN3 relocation is defective after gravity response. (c) Relative amount of seedlings with relocated PIN3-GFP in endodermal cells after gravity stimulation. (d) Defected PIN3 relocation after gravity stimulation. (e-g) PIN3 auxin mediated inner-lateralization is not altered in D_2.8.2 mutant. (g) Quantification of the PIN3-GFP fluorescence signal of the outer PM side of endodermal cells in hypocotyls in control situation and after NAA treatment.

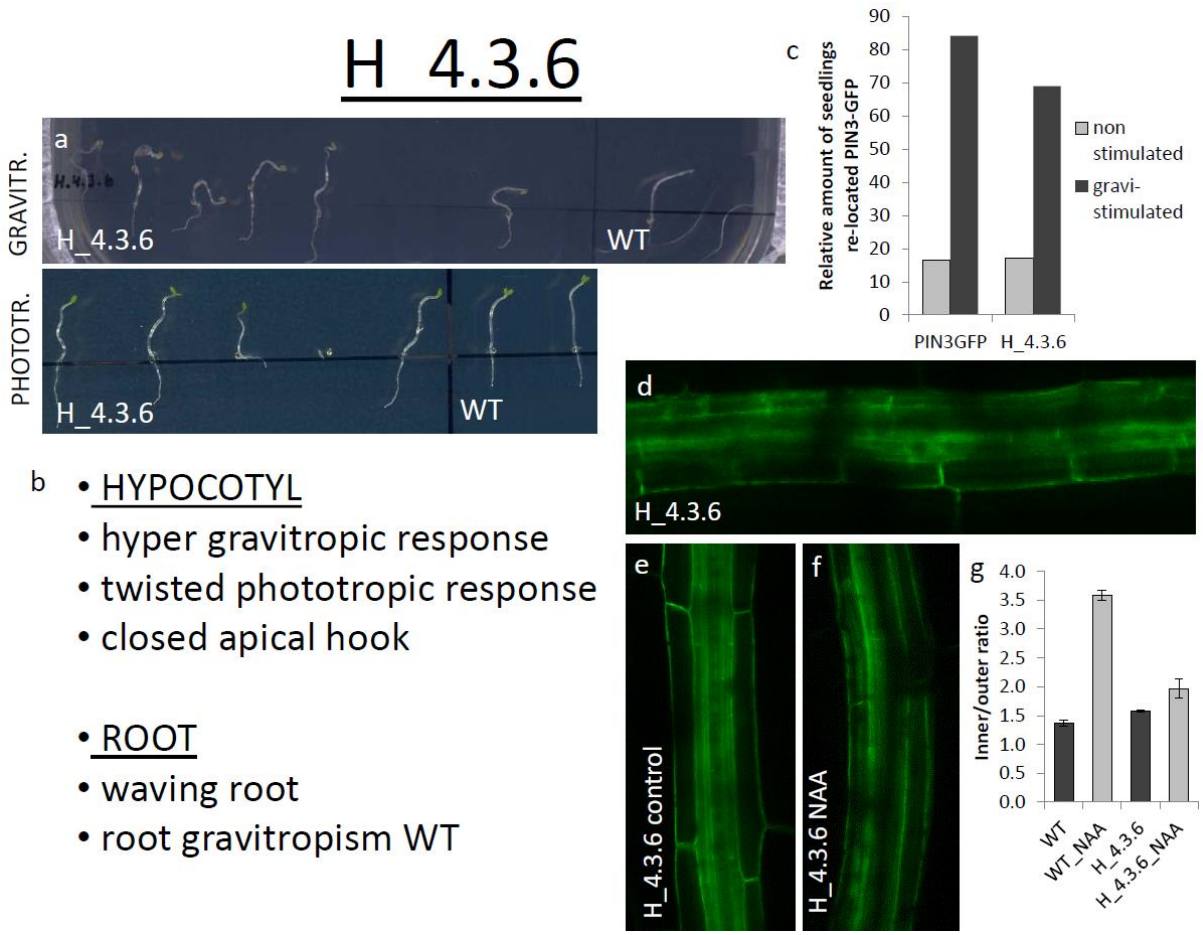


Figure 2. Mutant with hyper-bending phenotype from forward genetic screen.
(a) Mutant H_4.3.6 is hypersensitive to gravity and light stimuli. (b) List of mutant phenotypes. The growth of hypocotyls and roots is normal under light conditions and the apical hook is closed in the dark. (c-e) The PIN3 relocation is normal after gravity response. (c) Relative amount of seedlings with relocated PIN3-GFP in endodermal cells after gravity stimulation. (e-g) (g) Quantification of the PIN3-GFP fluorescence signal of the outer PM side of endodermal cells in hypocotyls in control situation and after NAA treatment.

A detailed bending response analysis and study of the PIN3-GFP relocation after gravity stimulation and after auxin treatment allowed the classification of the mutants into the two discussed subgroups. To identify the mutated genes, mapping populations were created by crossing the isolated mutants with the background line *PIN3::PIN3-GFP*. F2 seedlings showing the expected phenotype after gravity stimulation of etiolated hypocotyls were selected based on the same criteria as for the macroscopic primary screen. Thus far, whole-genome sequencing (Next-Generation Sequencing*;* NGS) was performed on nine selected mutants covering the five chromosomes of *Arabidopsis* (Table 1). We expect that the sequencing will lead to the identification of mutated nucleotides in specific genes that we anticipate to play a role during the PIN3-mediated tropic regulation.

Interestingly, the selected mutants belonging to the group with agravitropic hypocotyl phenotypes also displayed a defective gravity-induced PIN3-GFP relocation (data not shown),

but the mutants showing an overbending response were resistant to the auxin-mediated PIN3-GFP inner-lateralization (Figure 3). We hypothesized that these genes might be molecular components of pathways that regulate polar auxin transport and auxin accumulation via the regulation of the PIN3 polar localization in hypocotyls.

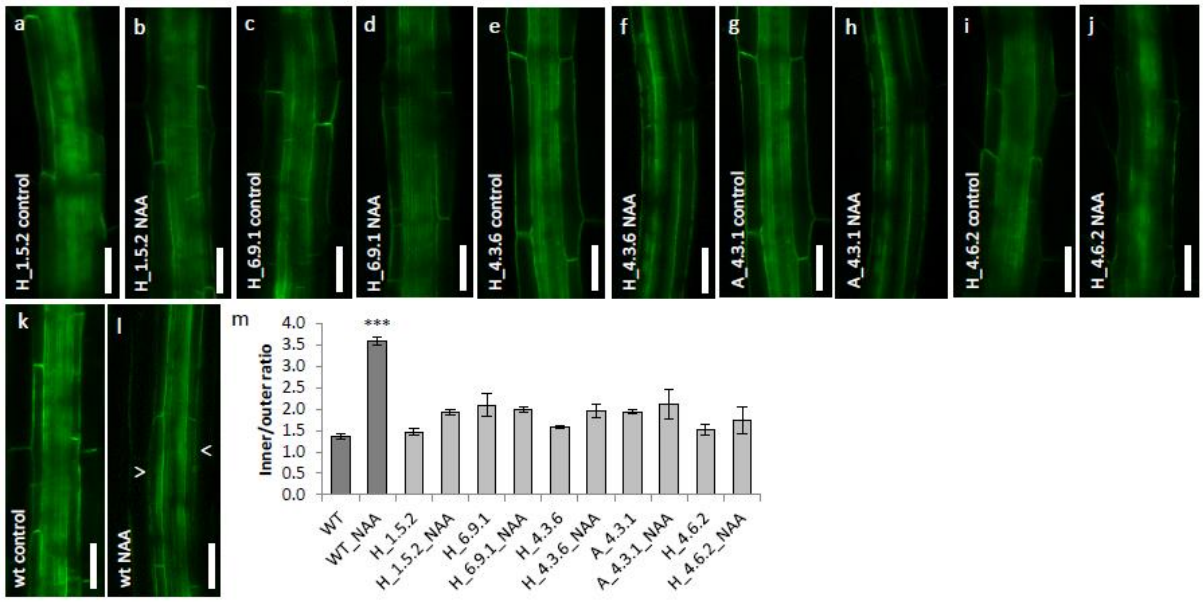


Figure 3. Auxin-induced PIN3-GFP inner-lateralization defects in selected mutants.
(a-l) The PIN3-GFP polar localization in hypocotyls of the H_1.5.2 (a), H_6.9.1 (c), H_4.3.6 (e), A_4.3.1 (g), and H_4.6.2 (i) mutants is comparable to that of the wild type (k). Auxin effects on PIN3-GFP polarity changes in the wild type (l) did not occur in the H_1.5.2 (b), H_6.9.1 (d), H_4.3.6 (f), A_4.3.1 (h), and H_4.6.2 (j) mutants. Arrowheads point to decrease of PIN3-GFP signal in the outer endodermal PM. Scale bars represent 50 µm. (m) Quantification of the PIN3-GFP fluorescence signal of the outer PM side of endodermal cells in hypocotyls in control situation and after NAA treatment. Error bars represent SE (Student *t*-test; *** *P*> 0.001).

After the whole-genome sequencing, we obtained a list of candidate mutations for each selected mutant. From this list, we selected mutations with a mutation frequency > 0.7 (Supplemental Figure 1) and mutations causing amino acid changes and open reading frame shifts, or introducing STOP codon within the coding sequences. The selected candidate genes for mutants D_2.8.2 and H_4.3.6 are presented in Supplemental Table 1. Within this list, we screened for mutated nucleotides in genes potentially playing a role during the PIN3 tropic regulation. Thus, they represent the best candidates as intracellular components that might be involved in the fine-tuning of the PIN3 PM polarity regulation to ensure a relevant auxin accumulation and subsequent proper bending response after environmental changes.

Table 1. Candidate mutant selected in the screen for PIN3 polarity regulators.

Name	Hypocotyl gravitropism	Phototropism	Apical hook	Root on light	Root gravitropism	NGS
H_4.3.6	Overbending	Twisted	WT like	Waving	WT like	Done
H_1.5.2	Overbending	Overbending	WT like	Longer	WT like	Done
D_2.30.3	Overbending	Overbending	WT like	Short	WT like	Ready
H_2.7.4	Overbending	No bending	WT like	WT like	WT like	Ready
H_6.9.1	Overbending	No bending	Open	Short	Slightly agravitropic	Ready
A_4.3.1	Overbending	No bending	Open	WT like	WT like	Ready
H_4.6.2	Overbending	No bending	Open	WT like	WT like	Done
D_2.8.2	Agravitropic	Slower bending	WT like	Short	Agravitropic	Done
D_4.9.9	Agravitropic	No bending	Open	WT like	WT like	Done

Detailed analysis of two mutants selected from the screen

Here, we describe in more detail the phenotypes of two mutants, one from category of agravitropic mutants (mutant D_2.8.2) and one with an overbending phenotype (H_4.3.6) (Table 1). Based on the plots from the whole-genome sequencing results, we selected possible intervals with increased mutation concentrations (see Supplementary Figure 1, intervals marked with green squares). From these intervals, only mutations with a frequency higher than 0.7 and only mutations causing amino acid changes or incorporating a STOP codon in the coding sequences were selected and confirmed by resequencing.

Mutant D_2.8.2 with an agravitropic phenotype carried mutations in the genes *AT3G48170*, *AT3G54220*, *AT3G59040*, and *AT3G61240* (Supplementary Figure 1). The most likely candidate is *AT3G54220*, namely *SCARECROW* (*SCR*) of which the mutant had been previously described to be agravitropic and to display a phenotype comparable to that of D_2.8.2 (Figure 4a,b; Fukaki et al, 1996; Fukaki et al, 1998). Similarly to a strong allele of *scr*, D_2.8.2 lacked an endodermal cell layer in roots and hypocotyls and instead exhibited a single cell file with mixed cortex/endodermal identity as described before (Figure 4c-h; Fukaki et al, 1996; Fukaki et al, 1998). The absence of an endodermal cell file questioned how the PIN3 polarity would be regulated in such mutant cells upon tropic stimuli or auxin treatment and whether this PIN3 polarity regulation was endodermis cell specific.

To evaluate in more detail the PIN3 polar localization in distinct hypocotyl cell layers, transversal sections of etiolated hypocotyls in the wild-type *PIN3::PIN3-GFP* line and in the D_2.8.2 mutant carrying a mutation in *SCR* were analyzed. An apolar PIN3-GFP localization was detected in endodermal cells of the wild-type seedlings (Figure 4e) and in the irregular cells

of the D_2.8.2 mutant (Figure 4g). In both cases, auxin induced PIN3 inner-lateralization after 4 hours of 10 µM NAA treatment (Figure 4f,h,i).

Next, we checked the cell specificity of the auxin impact on the PIN3 localization. The PIN3 signal is generally weaker in cortex cells than in endodermal cells of wild-type hypocotyls (Figure 4j). To avoid the PIN3 signal from the neighboring endodermal cells, only the outer lateral cortical cell side was measured and compared before and after NAA treatment. The fluorescence intensity of PIN3-GFP at the outer cortical cell side did not decrease after NAA treatment (Figure 4j,k) as it does in the endodermis (Chapter 3). This observation would support the hypothesis on the cell specificity of the NAA-dependent PIN3 polarity regulation. Cells with mixed identity in *scr* mutant are still able to change PIN3 polarity after NAA treatment but are not able to relocate PIN3 after gravity stimulation.

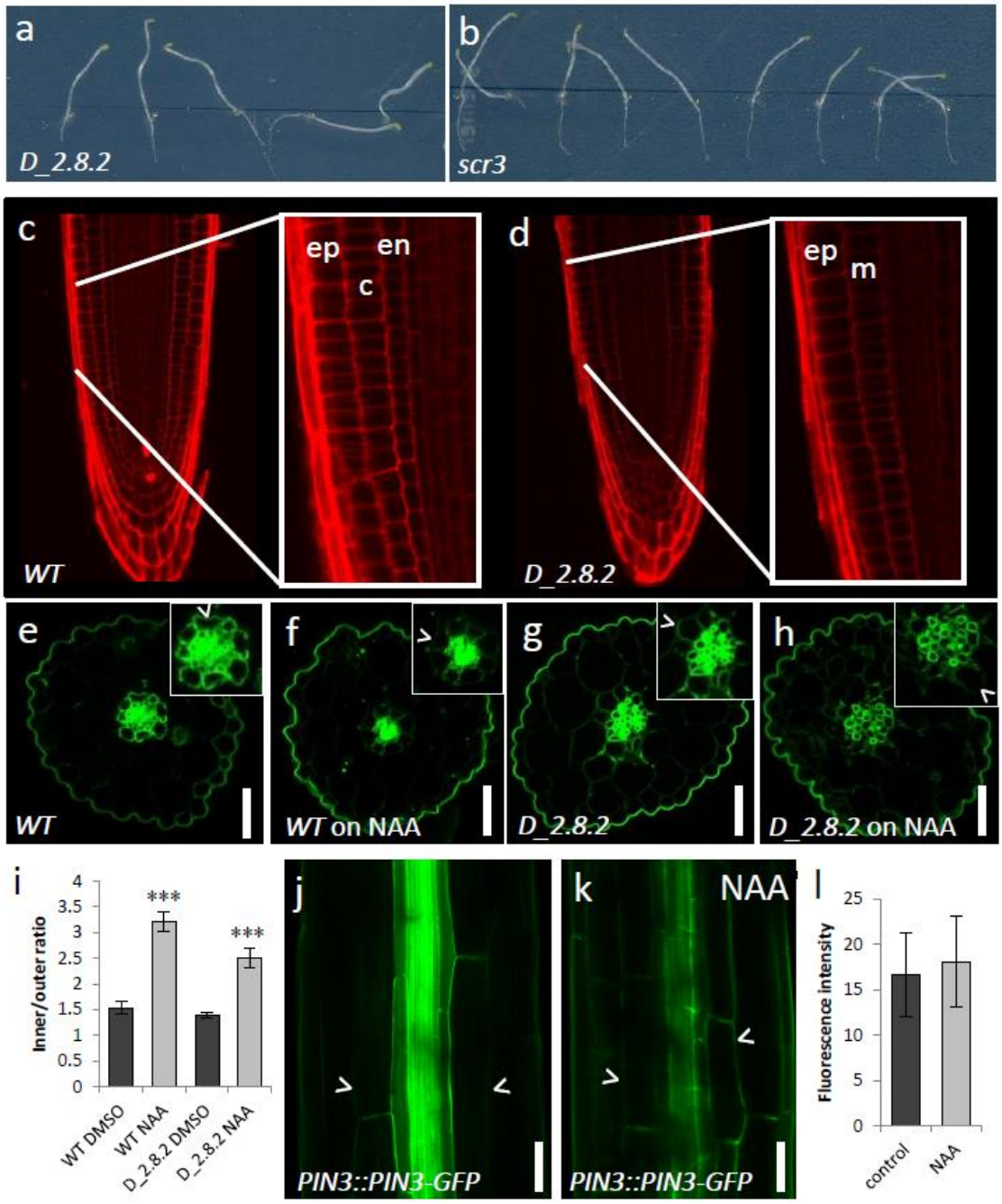


Figure 4. Cellular phenotypes of mutant D_2.8.2.
(a,b) Gravistimulated mutants D_2.8.2 (a) and *scr3* (b) showing comparable defective hypocotyl bending.
(c,d) Mutant D_2.8.2 lacking endodermal cell layer in roots (d) compared with the wild type (c). The mutant has only one layer of so-called ”mutant cells”. Abbreviations: c, cortex; en, endodermis; ep, epidermis; m, irregular mutant cell layer.
(e-i) Auxin-induced PIN3 degradation in mutant D_2.8.2. The PIN3 apolar localization in endodermal cells in wild type hypocotyls (e). Auxin-induced PIN3 degradation in wild-type endodermal cells (f). Mutant cells with apolar localization of PIN3 (g) and auxin-induced PIN3 degradation (h) in mutant cells in D_2.8.2 mutant. (i) Quantitative evaluation of PIN3 inner-lateralization in transversal sections. (Student *t*-test; *** *P*> 0.001). Arrowheads in zoom pictures point to the outer side of endodermal cells in the wild type (WT; e and f) and “mutant cells” in mutant D_2.8.2 (g and h).
(j-k) Auxin-insensitive response of cortical cells. PIN3-GFP signal at the outer side of cortical cells in wild-type hypocotyls (j). No change of PIN3-GFP signal in the cortex after 4 hours of 10 µM NAA treatment (k). (l) Quantification of fluorescence signal at the outer PM side of cortex cells in hypocotyls in control situations and after NAA treatments. Error bars represent SE. Arrowheads point at the outer PM side of cortex cells.
Scale bars represent 50 µm.

Mutant H_4.3.6, displaying an overbending hypocotyl phenotype (Figure 2), carried a mutation in gene *AT3G18780*, coding for the ACTIN2 (ACT2) protein. An independent mutant allele in *ACT2* from the T-DNA Salk collection (salk_048987) was analyzed and phenotypes were compared with those of mutant H_4.3.6. Both mutants displayed overbending responses (Figures 2 and 5c,f). *ACT2* is uniformly expressed in young seedlings and coexpressed in hypocotyls with *ACT7* and *ACT8* (McDowell et al., 1996a; McDowell et al., 1996b). To test the contribution of the actin cytoskeleton during the hypocotyl gravitropic response, a bending kinetic assay was performed in *actin* mutants with a predicted expression in hypocotyls, namely *act2*, *act7*, and *act8* (Figure 5). Only *act2* showed overbending phenotype.

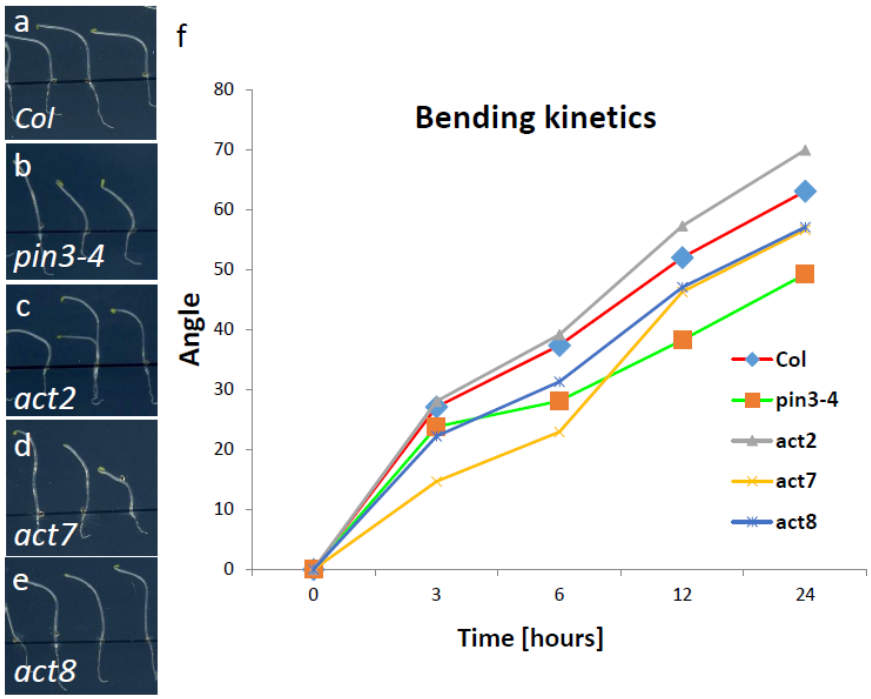


Figure 5. Actin role during gravity bending responses of hypocotyls.
(a-e) Bending responses of wild type (Col) (a), *pin3-4* (b), *act2* (c), *act7* (d), and *act8* (e) mutants after 24 hours of stimulation.
(f) Bending kinetics of wild type (Col), *pin3-4*, and *actin* mutants. Of the *actin* mutants, only *act2* displayed a slightly overbending response, whereas *act7* and *act8* had a reduced bending response.

Here we hypothesize that the PIN3 relocation might require microtubules (MTs) and/or the actin cytoskeleton. We used drugs that disrupt MTs, such as oryzalin, or actin filaments (MFs), such as latrunculin B (LatB). Both drugs altered hypocotyl gravity responses (Figure 6a and 6b). Oryzalin slowed down the response, whereas LatB first slow down and after prolonged time provoked a hypocotyl overbending. Gravity-induced PIN3-GFP relocation was inhibited by both drugs, with LatB having a stronger effect (Figure 6c-6e). In contrast, oryzalin, but not LatB, treatment inhibited auxin-induced changes of the PIN3-GFP polarity (Figure 6f-6j).

These data support the importance of actin and microtubule cytoskeleton during hypocotyl gravity responses.

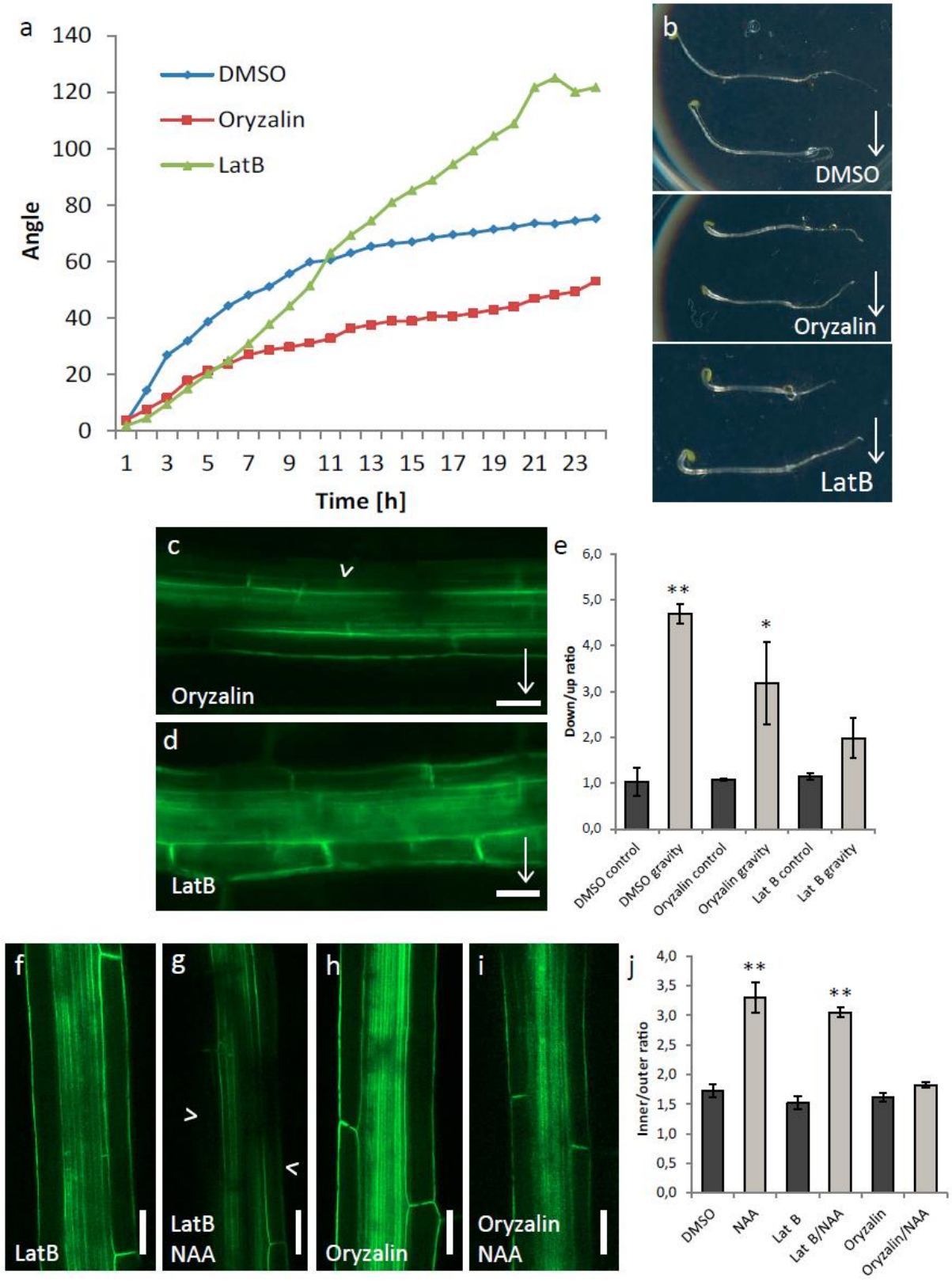


Figure 6. Gravity- and auxin-induced PIN3 relocation requiring MTs and actin filaments.
(a, b) Bending kinetics (a) of wild-type seedlings on oryzalin, LatB, and dimethyl sulfoxide (DMSO). Phenotypes of gravistimulated hypocotyls on DMSO, oryzalin and LatB (b).

(c-e) Oryzalin (c) and LatB (d) treatments inhibiting gravity-induced PIN3-GFP polarization. Quantification of the auxin-dependent PIN3-GFP relocation in endodermis cells (e). Graph shows the mean ratio of the inner-to-outer signal intensity. Error bars represent standard errors (Student's *t* test, * $P < 0.05$; ** $P < 0.01$).
(f-j) LatB pretreatment followed by LatB/NAA cotreatment causing PIN3-GFP inner–lateralization (g) compared to LatB treatment alone (f). Oryzalin treatment inhibited auxin induced PIN3-GFP inner-lateralization (i) in endodermis cells compared to treatment with oryzalin alone (h). Arrowheads mark PIN3-GFP depletion from the outer lateral side of endodermal cells. (j) Quantitative evaluation of auxin-dependent PIN3-GFP relocation in endodermis cells. Graph shows the mean ratio of the inner-to-outer signal intensity. Error bars represent standard errors (Student's *t* test, ** $P < 0.01$). Scale bars represent 20 µm.

Discussion

To identify novel molecular components of the PIN3 polarity regulation, we designed a forward genetic screen based on altered tropic responses of hypocotyls and on PIN3-GFP fluorescence imaging in *Arabidopsis thaliana*. After whole-genome sequencing, we selected several candidates as putative novel mutants carrying missense mutations that disrupted functions in the PIN3 polarity regulation and/or PIN3 stabilization. The genetic screen was designed accurately by taking into account both light- and gravity-induced signaling pathways and the auxin effect leading to PIN3 polarity changes in hypocotyls (Ding et al., 2011; Rakusova et al., 2011; Chapter 3). Thus far, we characterized nine candidates, of which seven with an overbending reaction to gravity stimulation and two with agravitropic phenotypes. Further phenotypic characterization of the selected mutants will be subsequently achieved by carefully analyzing the PIN3 localization and its regulation in different genetic and pharmacological contexts.

To validate that the candidate gene mutations in each of the nine mutants cause the phenotype of interest, we crossed (will cross) the mutants with independent alleles from various T-DNA collections with an insertion in the candidate genes. In case of recessive mutation, allelic tests by monitoring the gravistimulation responses by means of F1 seedlings of these crosses will confirm whether the candidate mutated gene is indeed the one causing the mutant phenotypes. In addition, phenotypic complementation of the mutants by introducing the functional gene in mutant plants will be performed.

A functional study of the confirmed molecular components involved in the PIN3 regulation will be done by analysis of publicly available loss-of-function alleles and overexpressing lines and by evaluating expression patterns and subcellular localizations. The intracellular localization of candidate proteins will be studied by translational fusions of the coding sequences with fluorescent reporters. Different promoters will be used to drive the fusion expression: endogenous, constitutive, and inducible promoters. The obtained vectors will be used to stably transform *Arabidopsis* plants. Transformants expressing translational fusions

with the red fluorescent protein (RFP) will be analyzed by confocal microscopy to provide insights into the (co)localizations of the possible regulators and PIN3-GFP proteins. To see the connection between the PIN3 relocation and tropic bending, the auxin accumulation in response to tropic stimuli will be studied in the isolated mutants by crossing in *DR5-GFP/GUS* auxin reporters (Friml et al., 2003).

One of the agravitropic mutants carried a mutation in *AT3G54220*, the *SCARECROW* gene. A strong *scr* loss-of-function mutant lacks an endodermal cell layer in roots and hypocotyls (Fukaki et al, 1996; Fukaki et al, 1998), but in the remaining cell layer with mixed endodermis and cortex identity an auxin-sensitive PIN3-GFP inner-lateralization was still observed. Moreover, auxin-induced inner-lateralization was shown not to be sequence specific by means of the PIN2, PIN3, and PIN7 proteins. Those PINs are undergoing inner-lateralization in hypocotyl endodermal cells after auxin treatment (see Figure S3 in Chapter 3). As PIN3 in the cortex does not respond to auxin application, cortical cells probably miss intracellular machineries to induce this process. This observation generates two hypotheses: (i) *PIN3* might be ectopically expressed when the plant lacks an endodermal cell layer and (ii) *PIN3* in the cortico-endodermal mutant cell layer would be unable to relocate after gravity stimuli, but would still respond to auxin for inner-lateralization. This might be, of course, caused by defected amyloplast formation and no gravity sensing in the cells. To test these two hypotheses, mutants without endodermal cell layer, such as *short-root* (Helariutta et al, 2000), and starchless mutants unable to sense changes in gravity vector (Vitha et al, 2007; Vitha et al, 2000), could be tested for *PIN3* expression and polar localization.

The overbending phenotype of H_4.3.6 mutant might be caused by a mutation in gene *AT3G18780, ACT2,* the only candidate conforming to the selection criteria of the mutations from the whole-genome sequencing (frequency > 0.7 and provoking amino acid change in the gene). Further confirmation of the mutant is currently under progress. Data supporting the hypothesis that mutant H_4.3.6 might be *act2* show that chemical depolymerization of actin microfilaments resulted in an overbending response (Figure 6a,b). Actin serves as a negative regulator of gravitropism, possibly by preventing a too fast sedimentation of statoliths (Nakamura et al, 2011; Yamamoto & Kiss, 2002), but the observed phenotypes hint at an opposite role for actin on the PIN3 relocation. The initial PIN3 relocation upon gravistimulation is defective when chemical inhibitors are used (Figure 6c-e). All together might explain the atypical bending kinetics of the wild type on the actin inhibitor when the initial bending is inhibited and an overbending is abruptly observed after a prolonged time of treatment.

However, more detailed analyses are needed to finally conclude on the role of the cytoskeleton during hypocotyl gravity responses and, more specifically, during the PIN3 polarity regulation.

Surprisingly, PIN3 in the endodermis of the H_4.3.6 mutant relocates correctly after gravistimulation and the *act2* single mutant does not show strong effects compared to the chemical inhibition of the actin filaments, implying genetic redundancy, probably with hypocotyls expressing *ACT7* and *ACT8*. The *act2/act7/act8* double and triple mutant combinations should answer this question and crosses are in progress.

These data, together with published work (Yamamoto and Kiss, 2002, Keuskamp, 2010) indicate that the cytoskeleton is involved in environmental responses in hypocotyls and, subsequently, induces PIN3 inner-lateralization after auxin treatment. Whereas the actin cytoskeleton might be a positive regulator of gravitropism during the gravity signal transduction stage (Nakamura et al., 2011), it might act also as an integral component of the cell growth mechanism during the gravity bending. The interpretation of the role of the cytoskeleton during tropic responses might be very complicated, because many different cellular processes (such as PIN intracellular trafficking, and cell elongation) need actin and MTs (Nick et al., 2009; Chen et al., 2014). Comparison of different *actin* and *MT* mutants for the PIN3 polarity regulation and cell elongation could be interesting in the future.

EXPERIMENTAL PROCEDURES

Plant material

The following transgenic and mutant lines were used: *DR5rev::GFP* (Friml et al., 2003); *PIN3::PIN3-GFP* (Žádníková et al., 2010); *scr-3*, (Fukaki et al., 1996), *actin2* (salk_048987; Nishimura et al., 2003), *actin7* (salk_131610; Salk collection), *actin8* (GABI_480B07; Salk collection). Mutant combinations with *DR5rev::GFP* and *PIN3::PIN3-GFP* were generated through genetic crosses.

Growth conditions

Seeds were sown on plates with half-strength Murashige and Skoof (½ MS) medium with sucrose agar and stratified at 4°C for 2 days. Germination was induced by exposing plates to light for 5-6 h before transfer to darkness and cultivation at 18°C for 4 days. For gravitropic stimulations, plates with 4-day-old seedlings were turned 90°. For confocal microscopy, a Zeiss confocal scanning microscope (Zeiss; http://www.zeiss.com) was used. To monitor gravitropic responses, plates were scanned 24 h after gravistimulation. Images were processed in Adobe Photoshop CS. Each experiment was done at least twice. Angles and fluorescence intensities were measured by ImageJ (NIH; http://rsb.info.nih.gov/ij). The fluorescence intensity rates of PIN3-GFP were measured as described (Rakusova et al., 2011). PIN3-GFP fluorescence intensity in hypocotyl transverse sections was measured usin ImageJ. Line was drown and compared outer and inner cell side of endodermal cel in WT and cells with mixed cortex/endodermis identity in D_2.8.2 mutant. Two replicates of at least 15 seedlings with synchronized germination were processed.

Pharmacological treatments

Wild-type seedlings were germinated and grown on vertical ½MS with sucrose agar plates at 18°C or 21℃ for 4 days. Treatments in the dark were done by transfer and incubation of 4-day-old etiolated seedlings on solid medium supplemented with NAA (10 µM, if not mentioned otherwise; Duchefa), latrunculin B (30 µM; Sigma-Aldrich), oryzalin (30 µM; Duchefa). All cotreatments with NAA were done after 1 h of pretreatment with the drug followed by 4 h of cotreatment with NAA or 6 h gravity stimulation. Control treatments contained an equivalent amount of solvent (DMSO; Sigma-Aldrich). For all comparisons, at least three independent experiments were carried out, giving the same significant results.

Real-time analysis

Gravity response of seedlings was recorded at 1 or 2-hour intervals for 1 or 2 days at 18°C with an infrared light source (880 nm LED; Velleman, Belgium) by a spectrum-enhanced camera (EOS035 Canon Rebel Xti, 400DH) with built-in clear wideband-multicoated filter and standard accessories (Canon) and operated by the EOS utility software. Angles of hypocotyls were measured by ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij). Minimum 20 seedlings with synchronized germination start were processed.

EMS mutagenesis of *Arabidopsis* seeds and mutant forward genetic screen

M2 seedlings, progenies of 2671 M1 0.3% EMS-mutagenized *Arabidopsis PIN3::PIN3-GFP* (ecotype Columbia-0) plants were analyzed by scoring the gravity bending response of hypocotyls and rescreened in M3 seedlings with the same criteria and observed under a confocal microscope for abnormal intracellular localizations of the PIN3-GFP signal in hypocotyls.

Whole-genome sequencing

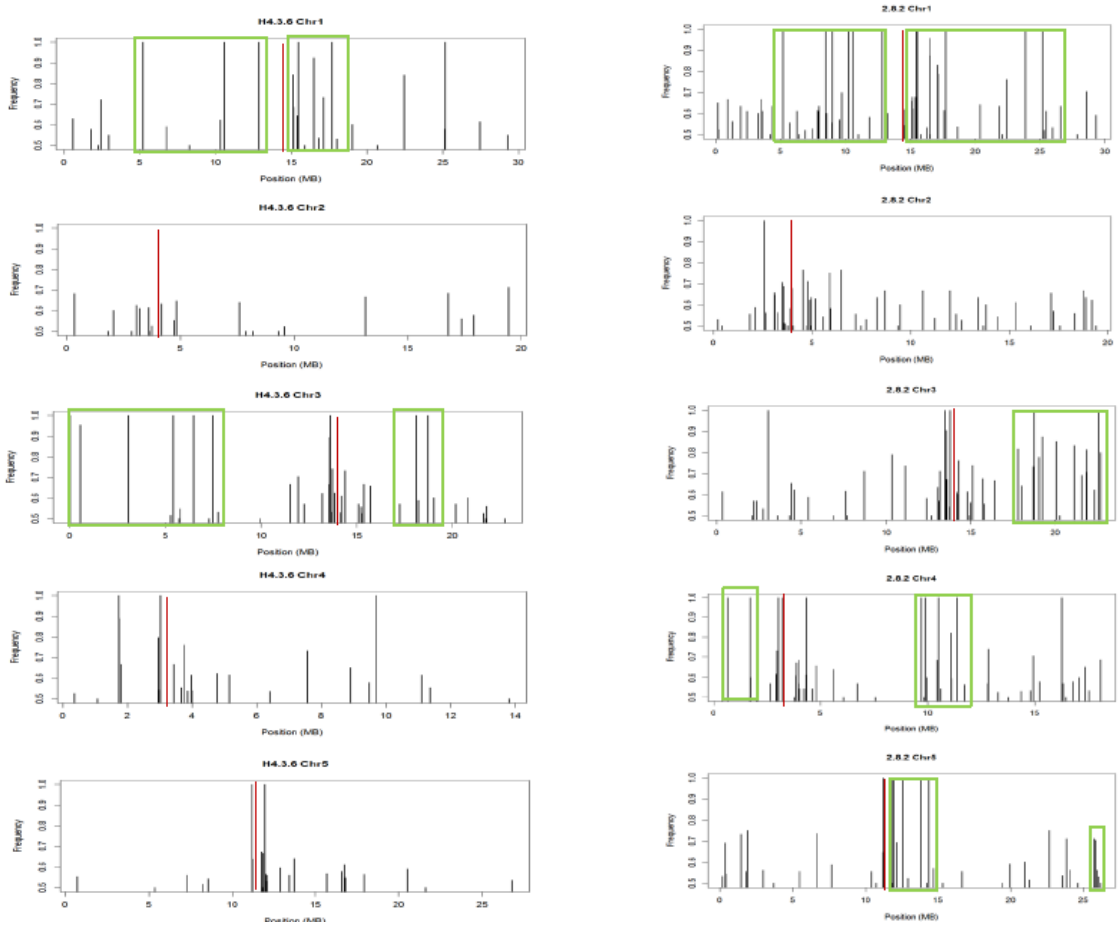
The identified mutants that displayed an altered gravitropic bending response and a defective PIN3-GFP relocation in the endodermal cells of hypocotyls were back-crossed into *PIN3::PIN3-GFP*. The population for the whole-genome sequencing was selected from a F2-segregating population. DNA from minimum 100 seedlings was isolated and sent for whole-genome sequencing (BGI, http://www.genomics.cn/en/index). The candidate genes with point mutations that introduced a STOP codon or caused amino acid changes were resequenced for confirmation. The list of candidate genes is presented in Supplementary Table 1.

Transverse sections

Four days old etiolated seedlings were fixed for 1 hour in 4% paraformaldehyde (Serva) in MTSB (50 mM PIPES, 5 nM EGTA, 1 mM MgSO4, pH=6,8) and immobilized in 5% (w/v) water solution of low-melting agarose (Sigma-Aldrich). Agarose blocks were mounted onto a Motorized Advance Vibroslice and 100-µm transversal sections through the hypocotyls were observed with a Zeiss 700 confocal microscope.

SUPPLEMENTAL INFORMATION

a **b**



Supplementary Figure 1. Plots for mutants H_4.3.6 and D_2.8.2 with the mutation frequency in the genome.
(a) Each plot of mutation's frequencies represents one chromosome of mutant H_4.3.6. (b) Each plot of mutation's frequencies represents one chromosome of mutant D_2.8.2. Green squares mark possible intervals with increased mutation frequency on the chromosome. Red lines indicate position of centromeres. Mutations common with the background line *PIN3::PIN3-GFP* were subtracted.

Supplementary Table 1. Mutated candidate genes of mutant D_2.8.2 and H_4.3.6.

Chromosome	Position	Mutation		Frequency	Gene	Description
Mutant D_2.8.2						
Chr3	17786891	GAC<AAC	D<N	0.77	AT3G48170	ALDEHYDE DEHYDROGENASE 10A9; ALDH10A9 aldehyde dehydrogenase (NAD) activity, chloroplast, cytosol
Chr3	20072023	CAA<TAA	Q<U	0.85	AT3G54220	SCARECROW, SHOOT GRAVITROPISM 1
Chr3	21823118	AGT<AAT	S<N	0.71	AT3G59040	biological process unknown, chloroplast, mitochondrion
Chr3	22668387	TCT<TTT	S<F	0.80	AT3G61240	DEA(D/H)-box RNA helicase family protein
Mutant H_4.3.6						
Chr3	6476082	TCT<->TTT	S<F	1.00	AT3G18780	ACTIN 2; actin that is constitutively expressed in vegetative structures

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Chapter 5.

ABP1 mediates coordination of cell and tissue polarities in Arabidopsis

Adapted from

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HR, KW, JK-V, M VM and JF initiated the project and designed most of the experiments. HR, MS, PG, EB, RDR, XC, SS conducted experiments and analyzed data. KW developed the computer model. All authors analysed and discussed the data. HR and KW wrote the manuscript and JF assisted manuscript writing.

ABP1 mediates coordination of cell and tissue polarities in *Arabidopsis*

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Abstract

Plant development largely relies on *de novo* establishment and modification of polarity axes but it remains conceptually unclear how individual cells acquire cues from their neighbours to achieve concerted polarization of the whole tissue. Here we propose a central role for the extracellular perception of the plant hormone auxin in cell and tissue polarization. Genetic analysis revealed that AUXIN-BINDING-PROTEIN1 (ABP1), which is localized in the Endoplasmic Reticulum (ER) and extracellular matrix, is required for both, the polarization of PIN auxin transporters in individual cells and auxin-mediated polarization of the whole tissues. ABP1 signalling influences local, asymmetric PIN membrane abundance thus mediating auxin effect on polar PIN distribution. This leads to canalization of auxin flow and propagation of the polarizing signal through the tissue. Computer simulations of this scenario predict the behaviour of *abp1* loss- and gain-of-function alleles and spontaneous emergence of PIN polarization observed during root patterning and in other canalization-related processes. Our study reveals previously unappreciated role of ABP1 in polarity and propose a plant-specific mechanism for the coordination of cell and tissue polarities during development.

Significance statement

A plant growth substance auxin is able to control its own cell-to-cell movement by modulating polar, subcellular localization of auxin transporters. Here we present insights into this feedback mechanism underlying auxin-mediated coordination of cell and tissue polarities in plants. Auxin as extracellular polarizing signal is perceived and interpreted by dedicated ABP1 auxin receptor at the cell surface. The proposed model that is grounded on the up-the-date experimental data is capable of reassembling the coordinated cell polarities from initially non-polarized situation. Experimental findings that validated computer model predictions indicate its plausibility and reveal a previously unknown mechanistic aspect of auxin effect on cell polarity and suggest various requirements of both nuclear and extracellular auxin perception for the proposed feedback mechanism. Together, experimental and computer modelling studies suggest a plant-specific framework for auxin-mediated polarization processes that guides developmental patterning in plants.

Introduction

A crucial question in developmental biology concerns how individual cells coordinate their behaviour based on the tissue context (Berleth & Sachs, 2001). Coordinated polarization of cells within tissue is central to the patterning of multi-cellular organisms. A prime example of this phenomenon is the synchronized polarization of cells during plant growth processes (Leyser, 2005; Traas, 2013). In plants, growth and formation of new organs is complicated by the fact that cells are kept at fixed positions within tissues by their rigid cell walls. Nevertheless, key developmental processes such as organogenesis and the formation or regeneration of vasculature do occur within pre-existing tissue structures and necessitate the specification of new, polar growth axes (Heisler et al, 2010; Marcos & Berleth, 2014; Reinhardt et al, 2003; Sauer et al, 2006; Scarpella et al, 2006). The plant-specific signalling molecule auxin can act as a tissue-polarizing signal, as it moves between cells in a directional manner. This directionality is determined by the polarized localization of PIN auxin efflux transporters in the plasma membrane of individual cells (Wiśniewska et al, 2006). A classical ‘canalization’ model has been formulated that envisions a positive feedback relation between auxin and its polar transport (Paciorek et al, 2005; Sachs, 1981). This feedback would be the underlying mechanism for a coordinated and self-organizing polarization of individual cells, ultimately leading to tissue polarity (Prusinkiewicz & Runions, 2012). Indeed, changes in PIN polar

localization in response to auxin were observed (Balla et al, 2011; Sauer et al, 2006), consistent with the canalization model, but the actual molecular and cellular mechanisms by which auxin mediates this effect remain poorly characterized.

Majority of current models attempting to explain the auxin-mediated polarization and resulting auxin canalization typically assume hypothetical auxin flux sensors or additional short-range signals which would report auxin levels of neighbouring cells. However, for none of these postulates a plausible biological basis has been established (Jonsson et al, 2012; Prusinkiewicz & Runions, 2012).

Here, we explore a putative mechanism for PIN polarization, relying on the combination of (i) intracellular auxin perception in individual cells, (ii) extracellular auxin perception by a receptor pool that is shared between adjacent cells and (iii) an extracellular auxin-mediated feed-back mechanism that regulates local abundance of auxin transporters at the membrane. Importantly, putative components of this model have been experimentally confirmed: The positive auxin effect on transcription of PIN auxin transporters (Peer et al, 2004; Sauer et al, 2006; Vieten et al, 2005) , mediated by the well-characterised SCF^{TIR1} nuclear receptor complex (Chapman & Estelle, 2009) and auxin effect on PIN abundance at the plasma membrane (Paciorek et al, 2005), which requires an extracellularly-localized ABP1 protein (Badescu & Napier, 2006; Jones & Herman, 1993; Tromas et al, 2010) regulating the clathrin-mediated PIN protein internalization (Robert et al, 2010). While it is known that ABP1 is of vital importance for numerous developmental processes (Braun et al, 2008; Chen et al, 2001; Tromas et al, 2009; Tromas et al, 2013), its role in cell and tissue polarity remains currently speculative.

Our genetic studies revealed an involvement of ABP1 in the auxin-mediated cell and tissue polarization. Revisited computer model integrating these findings correctly predicts polarity and auxin-mediated canalization defects in *abp1* loss- and gain-of-function mutants and accounts for the spontaneous emergence of steady-state tissue polarization from an originally non-polar situation.

Results

ABP1 localizes predominantly to the Endoplasmic Reticulum and the apoplast

The extracellular localization of ABP1 remains despite decades of studies a matter of debate. Earlier studies have demonstrated that *Zea mays* (Maize) ABP1 localizes to the endoplasmic reticulum (ER) as well as the extracellular space (Jones & Herman, 1993; Tian et al, 1995). Additionally, a wealth of physiological experiments that used agonistic and antagonistic ABP1 C-terminus peptides and anti-ABP1 antibodies demonstrated the extracellular action of ABP1 (Leblanc et al, 1999; Steffens et al, 2001). Similarly, in the model plant *Arabidopsis*, the extracellular localization of ABP1 was recently suggested by identification of its PM-localized receptor-like kinase partner (Xu et al, 2014).

To substantiate on these findings we further analysed *Arabidopsis* lines stably expressing ABP1-GFP fusion protein driven by its endogenous and constitutive 35S and RPS5 promoters. Previously described *35S::ABP1-GFP* plants (Robert et al, 2010) showed identical gain-of-function phenotypes (Xu et al, 2014) as plants expressing untagged *35S::ABP1*, supporting the functionality of the ABP1 overexpressor (Fig. S1). Consistent with studies from other species, *ABP1::ABP1-GFP* signal was detected predominantly at the ER (Fig. 1A) and confirmed by co-localization with representative markers for different subcellular compartments (Fig. S2). As the presence of ABP1 in the extracellular space is a key prerequisite in our hypothesis, we investigated ABP1 localization with three independent techniques. Firstly, we performed plasmolysis (Feraru et al, 2011) to detach the plasma membrane (PM) and cortical ER from the cell wall. This revealed a detectable signal of ABP1-GFP in the cell wall, in contrast to another ER-localized and not secreted Calnexin1-GFP, control (Fig. 1B, 1C). The comparison of secreted ABP1 with not secreted Calnexin1 supports that the observed signal associated with the cell walls does not originate from the ER. Secondly, we isolated total proteins from the culture medium of *Arabidopsis* cells expressing *35S::ABP1-GFP*. Immunoblot analysis revealed the presence of extracellular ABP1 similar to the positive control SKU5 (Sedbrook et al, 2002), a known apoplastic GPI-anchored protein but not our negative control, the PM-localized kinase PIP5K (Tejos et al, 2014) (Fig. 1D). Finally, we performed immunogold detection of endogenous ABP1 (using anti-ABP1 antibodies) visualized by transmission electron microscopy (see SI Appendix) also detecting the ABP1 signals in the apoplast (Fig. 1E). The almost complete disappearance of the immunogold signals following downregulation of ABP1 expression in the conditional *abp1* antisense line (ABP1 AS) (Fig.

1F) confirmed the specificity of the staining. These independent observations collectively confirm that ABP1 is present besides ER also in the extracellular space.

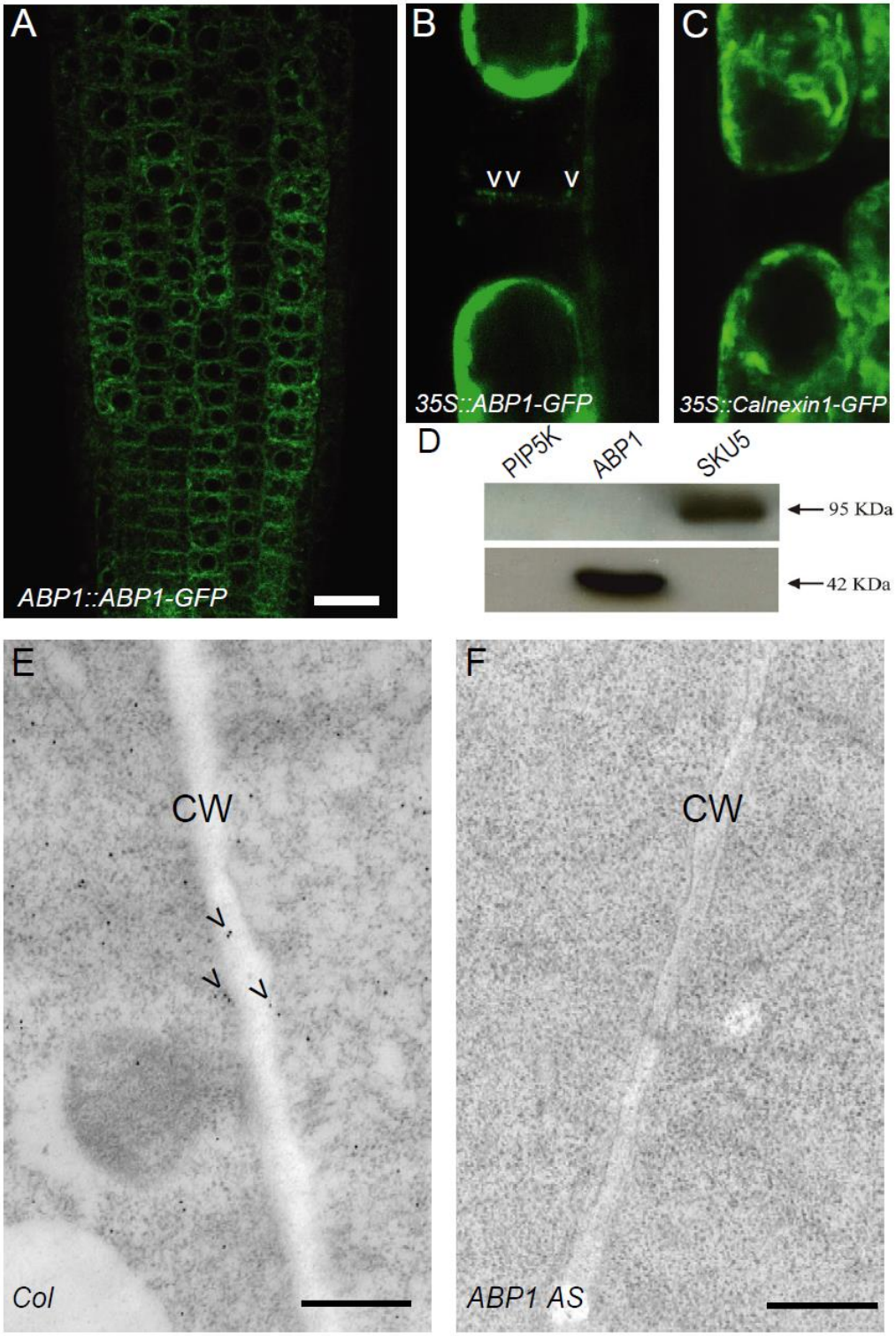


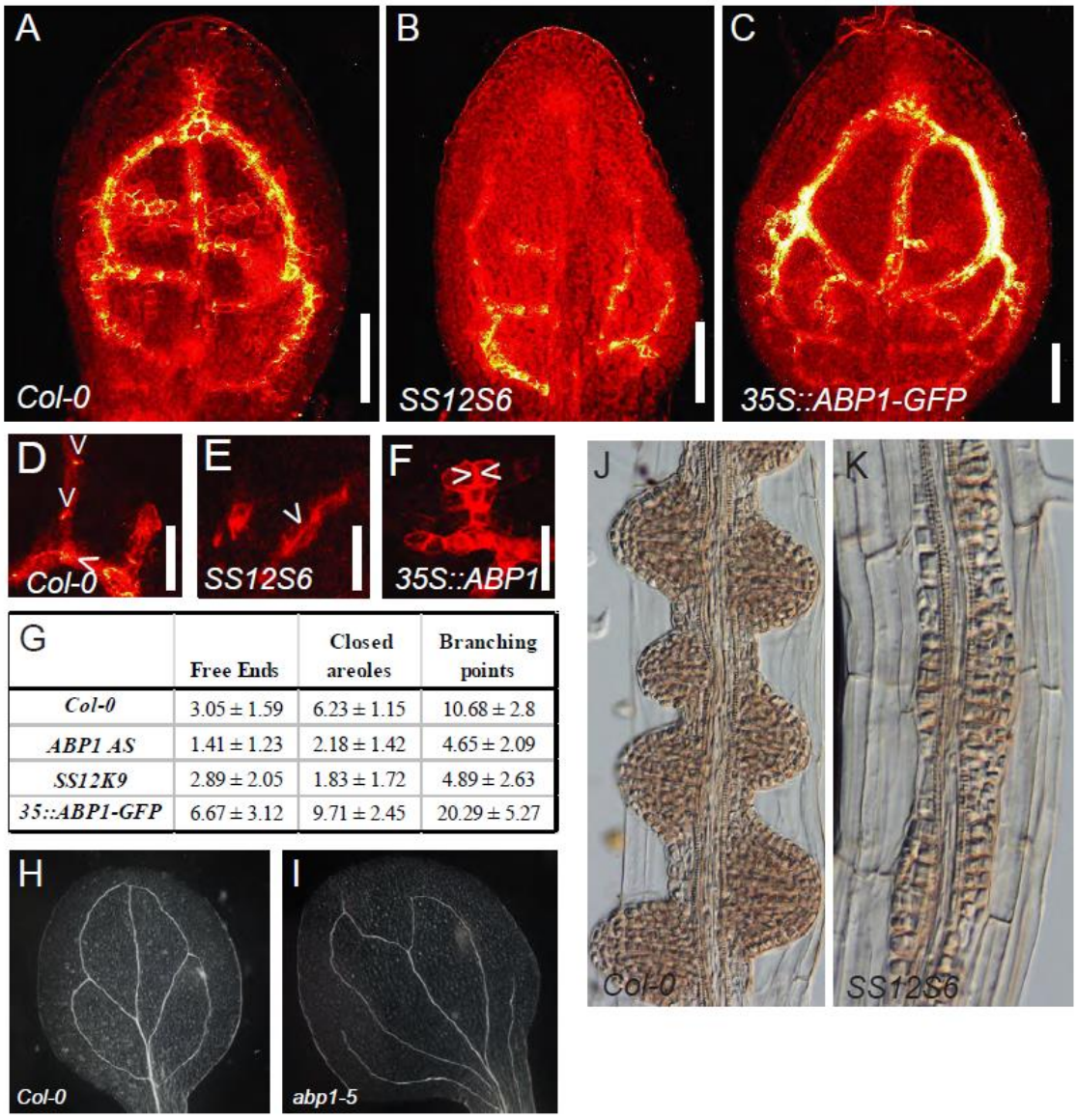
Figure 1. Localization of ABP1 in the endoplasmic reticulum and in the apoplast.
(A) *ABP1::ABP1-GFP* line expression in the root tip show predominant localization in endoplasmic reticulum**.** **(B, C)** Plasmolysis experiment treated with 0.4M mannitol for 30 min showed the ABP1-GFP signal in the apoplast (highlighted by white arrow heads, B) compare to Callnexin1 as ER localized but non-secreted protein (C). **(D)** Western-blot detection of ABP1secreted into the medium. PIP5K used as negative non-secreted control, SKU5 used as secreted positive control. **(E, F)** Transmission electron microscopy images of wild type *Col* (E) and ABP1

knock-down line (*ABP1 AS*) (F) incubated with anti-ABP1 antibody (highlighted by black arrow heads). Immuno-gold particles marking antigens in extracellular space and on cell surface are not present in *ABP1 AS* line after ethanol treatment.

ABP1 is required for an auxin-mediated patterning during plant development

ABP1 has been shown to be required plant embryogenesis, root and shoot growth as well as pavement cell shape (Braun et al, 2008; Chen et al, 2001; Xu et al, 2010). Next we investigated the role for ABP1 in others PIN (re)polarization- and canalisation-related processes, such as leaf venation patterning (Marcos & Berleth, 2014; Scarpella et al, 2006) and lateral root (LR) formation (Sauer et al, 2006).

Leaf vascular patterning is classical example of a process driven by the canalisation of auxin flow (Scarpella et al, 2006). The *abp1* knock-down lines showed alterations in the establishment of leaf vasculature that was characterised by a disconnected and less complex vascular network as compared to that of wild type leaves of the same age (Fig. 2A, 3B and 2G). Also, the *abp1-5* allele, containing a mutation in the ABP1 auxin binding pocket (Robert et al, 2010) displayed vascularization defects in cotyledons (Fig. 2H and 2I). In contrast, an *ABP1* gain-of-function mutant showed a visibly higher number of vein loops and more complex vein branching patterns (Fig. 2C, 2G) compared to the wild-type cotyledons of the same age (Fig. 2A, 2G). The size differences of leaves in *abp1* mutants are presumably caused by the additional ABP1 role in cell growth regulation (Chen et al, 2001). Consistently, coordinated PIN1 polarization associated with the venation pattern (Sawchuk et al, 2013; Scarpella et al, 2006) (Fig. 2D), was compromised as evident from nonpolar or bipolar PIN1-GFP signal in developing leaf vasculature cells of *abp1* knock-down and overexpression lines (Fig. 2E, 2F).



G	Free Ends	Closed areoles	Branching points
Col-0	3.05 ± 1.59	6.23 ± 1.15	10.68 ± 2.8
ABP1 AS	1.41 ± 1.23	2.18 ± 1.42	4.65 ± 2.09
SS12K9	2.89 ± 2.05	1.83 ± 1.72	4.89 ± 2.63
35::ABP1-GFP	6.67 ± 3.12	9.71 ± 2.45	20.29 ± 5.27

Figure 2. Cell polarization and development defects in *abp1* mutants.
(A-C) PIN1 antibody detection (α-PIN1) during venation patterning in *Arabidopsis* young leaves in *wild-type* control (A), *SS12S6* (B) and *35S::ABP1 -GFP* (C) mutant lines. Scale bar: 50 µm. **(D-F)** Magnification of higher order leaf vein the leaf of *wild-type* (D), *SS12S6* (E) and *35S::ABP1–GFP* (F) mutants showed an ectopic/apolar PIN1 localization. **(G)** Quantification of leaf venation patterning defects in ABP1-related mutants. Numbers represent the mean frequency ± SDs of venation features (free venation ends and vein branching points) per each sampled young leaf (n>100). White arrowheads depict preferential PIN1 polar localizations. **(H, I)** Cotyledon in *abp1-5* mutant (I) exhibit vascularization breaks in a higher frequency (18 %) compare to *wild type* (3 %) (H). **(J, K)** Lateral root phenotype of 7-10-day-old *wild-type* (J) and *SS12S6* plants (K). After auxin incubation (6 hours in 10 µM NAA), the initiation of LR occurred normally in *wild-type* plants (J) and was delayed or compromised (fused primordia) in the *SS12S6* mutant (K).

We also examined synchronized LR formation following exogenous auxin application – a process known to require coordinated changes in PIN polarities and rearrangement of auxin maxima (Benkova et al, 2003; Sauer et al, 2006). The *abp1* knock-down roots were strongly impaired in the coordinated cell divisions leading to the formation of LR primordia (Fig. 2J, 2K). This phenotype is reminiscent of mutants in the polar auxin transport (Benkova et al, 2003)

or polarization components (Kleine-Vehn et al, 2008). Similarly, ABP1 gain-of-function lines show a number of different developmental defects whose strength correlated (Fig. S3A-S3H) with ABP1 expression levels in several ABP1 overexpression lines (Fig. S3I).

These observations collectively show that balanced ABP1 activity is required for multiple developmental processes including those typically requiring auxin canalization.

ABP1 is required for PIN polarity and auxin distribution in the root

A role of ABP1 during the vasculature formation, LR development and other processes dependent on canalization of auxin flow as well as PIN polarity defects in vasculature prompted us to test ABP1 involvement in auxin-mediated polarization. We examined PIN polarization and auxin distribution in conditional *abp1* knock-down (*ABP1* antisense, *SS12S6* and *SS12K9*) (Braun et al, 2008), constitutive gain-of function (*35S::ABP1-GFP*) (Robert et al, 2010) and (*RPS5::ABP1-GFP*) as well as conditional gain-of-function (*XVE>>ABP-GFP*) lines. The *abp1* knock-down lines displayed largely undisturbed pattern of PIN localization in the root meristem (compare Fig. 3A to Fig. 3B) that is consistent with previous observations (Tromas et al, 2009). On the contrary, ABP1 overexpression lines showed partial repolarization of PIN proteins that was manifested by PIN lateralization in both endodermis and cortex cells of the root (Fig. 3C).

Collectively, our *in planta* findings suggest that the balanced amount of ABP1 is necessary to maintain PIN polarities. Moreover, these findings also clarify that PIN polarity once established it remains difficult to disrupt. However, an increase of ABP1 levels can lead to partial PIN re-polarization, depending on the developmental context.

ABP1 mediates the auxin effect on PIN polarity

An easily testable example of the auxin effect on cell polarity is the basal-to-lateral relocation of PIN proteins in roots following exogenous auxin applications (Sauer et al, 2006) (Fig. 3D and 3G).

The *abp1* knock-down lines following ABP1 down-regulation showed pronounced defects in auxin-mediated PIN lateralization (Fig. 3E,3G). On the contrary, increased ABP1 levels (in ABP1 overexpression lines) showed strong PIN lateralization, regardless of auxin applications (Fig.3C,3F). We speculate that ABP1 overexpression increases constitutive recycling of PIN proteins from the preferential polar side, causing increase of intracellular PIN pool and subsequent delivery of PINs to lateral membrane segments.

Collectively, our data indicate that ABP1-mediated signalling is required for auxin–mediated PIN repolarization in roots.

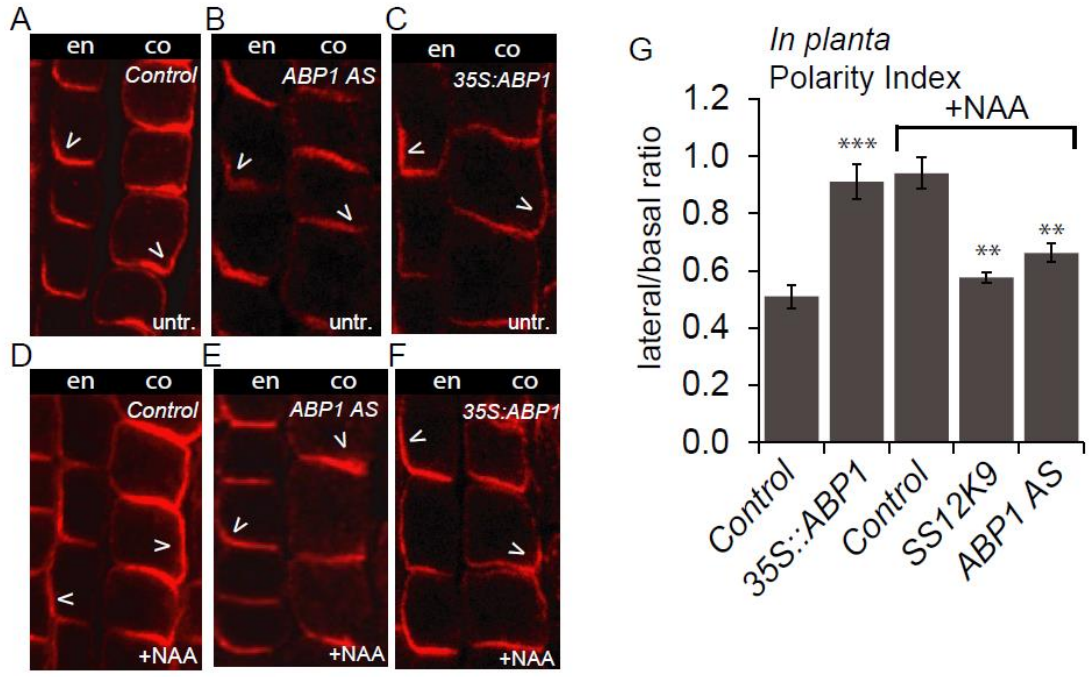


Figure 3. Experimental observations of auxin-mediated, ABP1-dependent PIN polarizations.
(A-F) Experimentally observed PIN polarities in the root meristem without and with exogenous auxin applications for control (A and D), ABP1 AS (B and E) and less pronounced basal polarization in ABP1 overexpression line (35S::ABP1(-GFP)) (C and F)**.** Plants were treated with 10µM NAA for 4 hours. Abbreviations for root cell types are (en) endodermis; (co) cortex.
(G) Quantifications of basal-to-lateral PIN1 ratio observed in experiments for control and ABP1 gain-of-function displayed a similar tendency after auxin application in endodermal cells. Auxin effect was compromised in ABP1 loss-of-function mutants. Error bars are SEs (Student's t-test, **P < 0.01; ***P < 0.001). Number of samples was n>10. Abbreviations for root cell types are (en) endodermis; (co) cortex.

Extracellular ABP1 model integrates a feedback between ABP1 and PIN internalization

The extracellular localization (Fig. 1) and action (Leblanc et al, 1999; Steffens et al, 2001) of ABP1 along with its requirement for different canalization-dependent processes (Fig. 2) as well as auxin effect on PIN polarity in root cells (Fig. 3) prompted us to revisit our earlier model of extracellular auxin perception-mediated canalization (Wabnik et al., 2010).

Computational modelling approaches has been instrumental in providing insights into mechanisms of auxin-mediated development (Bennett et al, 2014; Jonsson et al, 2012; Prusinkiewicz & Runions, 2012; van Berkel et al, 2013). To test *in silico* whether the extracellular auxin signalling mediated by ABP1 could suffice for generating and maintaining PIN polarities, we integrated a feedback mechanism between ABP1 and PIN polarity in the computer model, referred to as the 'extracellular ABP1' model (Fig. 4A). This model is based

on previously introduced concept of extracellular auxin perception that is coupled to the regulation of PIN internalization thus controlling capacity of auxin flow across the plasma membrane at the different cell sides (Wabnik et al, 2010). The extracellular ABP1 model also integrates experimental data on nuclear auxin signalling regulating PIN transcription and thus overall PIN-mediated auxin efflux capacity (Petrášek et al, 2006) (Fig. 4A; shown in red) and auxin influx by AUX/LAX family of auxin influx carriers (Peret et al, 2012) (Fig. 4A; shown in yellow). The apoplast is represented as two adjacent cell surfaces of neighbouring cells. The model assumes that neighbouring cells compete for a common pool of ABP1 by responding to differences in auxin concentration at opposite cell surfaces resulting from the net auxin fluxes across the membranes of neighbouring cells (Fig. 4A). However, the key upgrade of the 'extracellular ABP1' model (Fig. 4A) over the previous approach (Wabnik et al, 2010) (Fig. S4A) lies in the implementation of the extracellular auxin signalling. The 'extracellular ABP1' model explicitly integrates the experimentally supported finding that free ABP1 (Fig. 4A; black box) positively regulates the clathrin-mediated protein internalization from the cell surface (Fig. 4A; black arrows) (Robert et al, 2010) whereas previous model hypothesized an apoplastic auxin receptor that acts as an inhibitor of PIN internalization (Fig. S4A) (Wabnik et al, 2010). Thus these two models present alternative realizations of extracellular auxin signalling. Since in our model, ABP1 acts generally on both influx and efflux auxin carriers internalization, we speculate that the ABP1 effect on auxin influx carriers is counteracted by higher lateral mobility of auxin influx carriers within membrane (Kleine-Vehn et al, 2011) (see SI Appendix) which would then lead to uniform auxin influx into the cells (Fig. 4A). This assumption was necessary for robust model predictions (Fig. S4C).

According to our computer model, uniform auxin influx combined with high auxin efflux from Cell 1 and low auxin efflux from Cell 2 in our model (Fig. 4A; source-sink relation) would capture more free ABP1 on the surface of Cell 2 (more auxin enters the Cell 2 than leaves it). Trapped ABP1 would participate in signalling whereas auxin-bound ABP1 freely diffuses within the apoplast and eventually dissociates. The differences in ABP1 signalling between adjacent cells are reflected in the ratio of auxin-bound ABP1 to free ABP1 (Fig. 4B). The lower this ratio the more PIN proteins are internalized from the PM (Fig. 4A). Proposed model initially requires at least 25% auxin gradient across the apoplast to break symmetry of free ABP1 signalling, which is smaller than the previous version lacking ABP1-specific assumptions (Fig. S4B; compare solid and dashed lines, respectively). To substantiate on these observations, we performed more detailed model parameter sensitivity analysis with commonly used mathematical technique called bifurcation analysis (Fig. S5). Our model was generally

robust to parameter perturbations (Fig. S5A-S5C) unless large values of apoplastic auxin diffusion (Fig. S5B) and free ABP1 diffusion rates (Fig. S5A) were used.

Extracellular ABP1 model predicts auxin canalization

Next we tested whether our model could reproduce coordinated PIN polarization observed in auxin-canalisation related processes, such as leaf vasculature formation and tissue regeneration (Sauer et al, 2006; Sawchuk et al, 2013; Scarpella et al, 2006). We run model simulations on a cellular grid that incorporated an auxin-producing cell (auxin source, green bar; Fig. 4C) and an auxin-degrading cell (auxin sink, blue bar; Fig. 4C) at opposite boundaries. 'Extracellular ABP1' model reassembled the establishment of a polarized auxin canal connecting source and sink as defined by predicted PIN polar localizations (Fig. 4C). Notably, our model recapitulated inner-lateral PIN localization of cells directly adjacent to the auxin channel and gradual fade away of PIN expression at an increasing distance from the channel (Fig. 4C) that mimics observations *in vivo* (Sawchuk et al, 2013; Scarpella et al, 2006) or following an application of an artificial auxin source (Balla et al, 2011; Sauer et al, 2006). These model predictions were largely insensitive to a particular choice of auxin source/sink locations, yet the sink location determined the preferential direction as well as the shape of the auxin channel (Fig. S6A-S6C).

The consistency between predicted and experimentally observed PIN polarization during leaf vein pattern development suggest that the proposed ABP1-mediated extracellular perception mechanism may also account for PIN patterns observed during regeneration of vasculature after wounding.

Extracellular ABP1 model predicts polarities and auxin distribution in the root meristem

Next we tested how our model performs in a more complex scenario such as root meristem patterning that strongly depends on multiple PIN proteins mediating a network of an auxin fluxes stabilizing auxin maximum at the centre of the meristem (Blilou et al, 2005; Friml et al, 2002a). Available computational models of root development typically rely on fixed PIN polarities derived from experimental observations (Band et al, 2014; Grieneisen et al, 2007; Jones et al, 2009; Mironova et al, 2010; Swarup et al, 2005) and thus do not address how these PIN polarities are established or maintained. To address this issue, we performed model simulations (Fig. 2D- 2F) on a virtual root system build on the simplified 2D cellular template consisting of essential root tissues (stele (st), epidermis (ep), endodermis (en) and cortex (co)). We introduced an auxin source in the uppermost row of stele cells imitating the auxin unloading

from the vasculature to proximal root regions (Swarup et al, 2001) (Fig. 2D; green horizontal bar). Additionally, auxin can exit the *in silico* root through epidermal cells at the top to mimic the connection to root-shoot interface which was not modelled (Fig. 2D, blue horizontal bars).

Model simulations started from the non-polar, homogenous situation (no bias for particular PIN localizations) and predicted a basal (rootward) PIN polarization in the stele (Fig. 4D), similar to that of the auxin canalization simulations (Fig. 4C). Following auxin accumulation at the root tip (Fig. 4D), auxin transport spontaneously reversed as shown by the apical (shootward) PIN localization in the epidermal cell files, driven by *de novo* PIN polarization from the secondary local auxin source at the root tip (Fig. 4D, 4E). In time, basal PIN polarity in vascular cell layers (Fig. 2F; middle panel) and apical PIN polarization in epidermal cells (Fig. 2F; lower panel) caused a transient auxin circulation via cortex, endodermis and pericycle cells (Fig. 4D) and led to the stable auxin maximum in the distal part of the root meristem (Fig. 4E). These predicted PIN localizations, such as basal PIN1 localization in inner stele cells and apical localization of PIN2 in epidermis cells are observed *in planta* (Fig. 4F) (Blilou et al, 2005; Friml et al, 2002b). Our model also predicted inner-lateral spreading of basal PIN polarization in endodermis and outer-lateral spreading of apical PIN localization in cortex cells, which also are found *in planta* (Fig. 4F; upper panel). However, the 'Extracellular ABP1' model predicted the strong lateralization of PIN protein in cortex/endodermis cells adjacent to the root tip (Fig. 4E) that was not found *in planta*. This preferential PIN lateralization in the proximity of the root tip occurred presumably because strong fluxes of auxin through stele and epidermis attract adjacent cortex/endodermis cells to polarize towards the high auxin concentrations as observed in the simulations of auxin canalization (Fig. 4C). We concluded that discrepancies between the predicted and *in planta* PIN polarities may result for the absence of additional, yet to be identified components of PIN polarity regulation in the root meristem.

Taken together, the extracellular ABP1 model was capable of reproducing, in a self-emergent manner, key aspects of a PIN polarization network (Fig. 4F) in the root from the originally non-polar situation. Interestingly, the apical and basal polarities were generated by the minimalistic model without incorporating additional polarity mechanisms such as PINOID-mediated PIN phosphorylation that are known to stabilize PIN polarities *in planta* (Michniewicz et al, 2007).

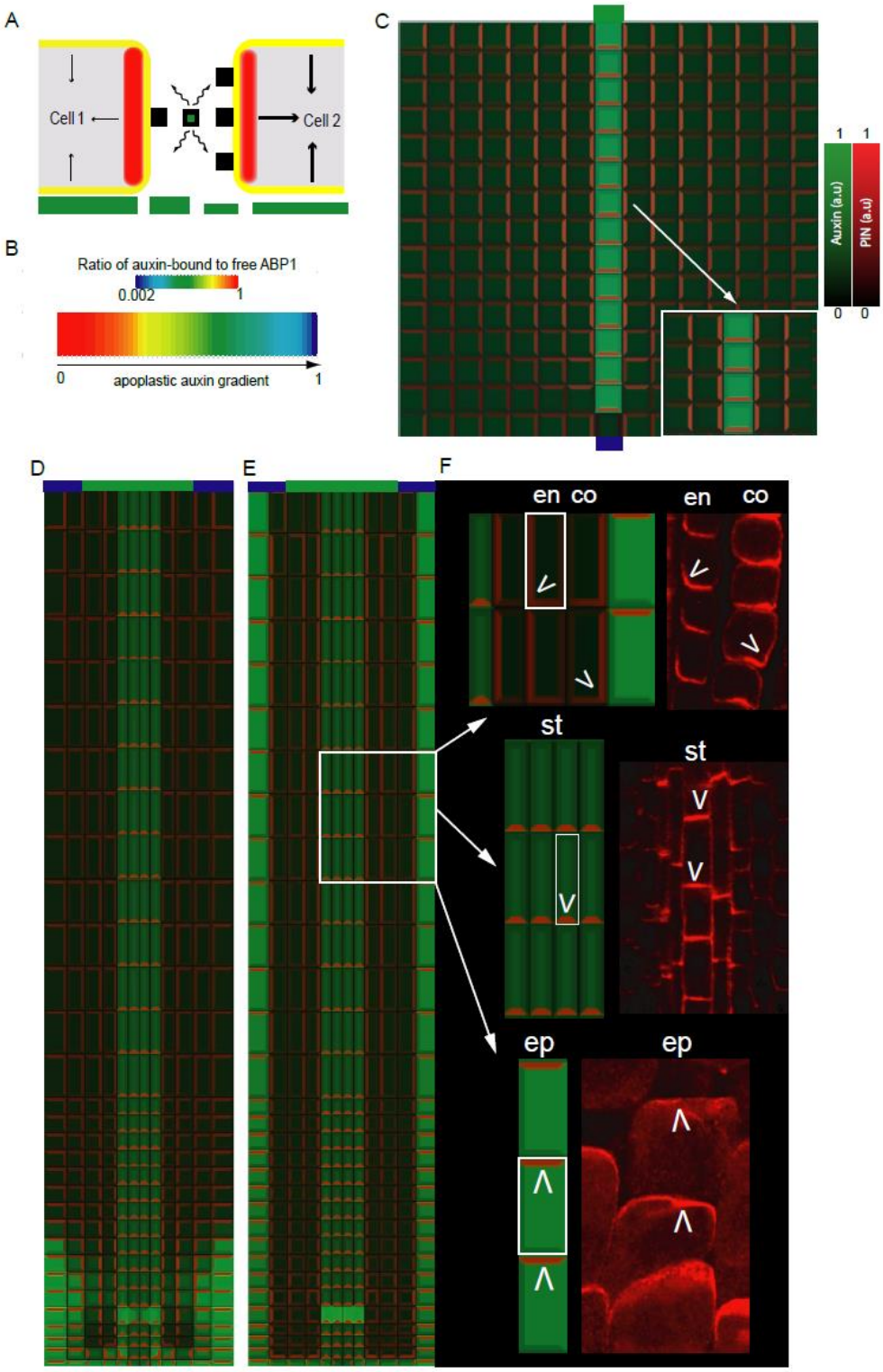


Figure 4. Computer simulations predict *in planta* PIN polarization and auxin distribution.
(A) The extracellular ABP1 model scheme integrates a positive effect of free ABP1 (a box) on the effective rate of PIN protein internalization (black arrows). Auxin-free ABP1(signal) is represented by black box and auxin-bound ABP1 is represented by a green box with black borders and is uniformly distributed in the apoplast due to free diffusion (shown as zigzag arrows). Symbolic auxin gradient is shown with green bars. **(B)** Representative ratio of auxin-bound ABP1 to auxin-free ABP1 for Cell 2 (inverse measure of PIN internalization in [a.u] colour coded; the lower the ratio the stronger diffreneces in PIN internalization rates) as the function of apoplastic auxin gradient, such that 1 corresponds to 100% gradient [a.u]. Note that ~25% gradient results in visual difference in PIN internalization rates (ratio < 1) of Cell 2 and source Cell 1 . Note that the extracellular ABP1 model (Fig. 2A)

displays a high dynamic response to apoplastic auxin concentrations. ABP1 acts on both PIN proteins and influx carriers. This effect is masked for influx carrier based on the assumption of fast lateral diffusion of these proteins compared to PIN proteins. **(C)** Simulation of auxin flux canalization preceding the venation patterning in plants obtained with the extracellular ABP1 model. Colour coding map for predicted auxin concentrations (green) and PIN levels (red). **(D, E)** Time-lapse computer simulations predict sequential establishment of *in planta* patterns of PIN and auxin distributions in the root meristem. **(F)** PIN polar localizations predicted by the model (left panel) and those observed experimentally using anti-PIN1 and anti-PIN2 immunolocalizations in the *Arabidopsis* roots (right panel). Abbreviations for root cell types are (en) endodermis; (co) cortex; (st) stele.
Horizontal blue bars show the position of the auxin sink (C) or alternatively auxin exit from the virtual root (D, E) whereas horizontal green bars depict the site of auxin source (C) or auxin influx in the root (D, E).

Simulations of *abp1* mutants predict alterations of auxin distribution and PIN polarity

Our experimental data and model simulations suggest that coupling extracellular ABP1-mediated signalling to PIN internalization rates can translate the auxin distribution within tissues into the coordinated polarization of PIN-mediated auxin transport in individual cells.

Next we tested sensitive is the steady state PIN polarization pattern, once established, to reduction of ABP1 activity (by 100-fold). Model simulations displayed no dramatic change in PIN localizations (compare Fig. 5A and 5B) that is consistent with our experimental observations (Fig. 3B). These model predictions result from a global decrease in PIN internalization at all sides of the virtual cells (by globally reducing ABP1 activity), which rather stabilizes but not alters already established PIN localization pattern. Despite no dramatic change in PIN polarities, the simulations predicted that the auxin maximum in the root tip was less focused compared to that of the 'WT' simulations (compare Fig. 5A and 5B). This additional model prediction could be subsequently tested in future studies.

In complementary simulations, we tested an effect of ABP1 overexpression. A 100-fold increase of ABP1 in the model severely enhanced PIN internalization from the preferential polar side of the cells supplementing the intracellular PIN pool with recycled material that is subsequently delivered to lateral cell sides leading to the lateralization of PIN proteins in cortex and endodermal tissues (Fig. 5C), exactly as observed in our experiments (Fig. 3C) . Nevertheless, further increase of ABP1 led to the loss of PIN polarity (data not shown) because the severe inbalance between PIN exocytosis and PIN endocytosis.

Taken together, model simulation of ABP1 loss- and gain-of-function mutants are consistent with experimental observations and together indicate that balance of ABP1 levels is essential for maintaining proper PIN polarity network in plant roots.

Simulations of auxin application predict auxin-mediated PIN re-polarization

Our experimental data suggest that auxin-mediated repolarization/lateralization of PIN proteins in root cortex and endodermis cells requires ABP1-dependent signalling (Fig. 3D-3F).

Therefore, we investigated if our computer model of the root meristem can recapitulate this auxin-driven PIN lateralization. Simulations of virtual auxin production in every cell of the root led to higher auxin fluxes in stele and epidermis and repolarization of cortex/endodermis cells towards these two main transport streams thus separating them more efficiently (Fig. 5D, 5G). These simulations fairly agree with the experimentally observed specific spatial aspects of repolarization in different cell types (Fig. 3D) (Sauer et al, 2006), namely inner lateralization in endodermis and pericycle cells and outer lateralization in cortex cells (Fig. 3D). These results show that our model can predict known auxin effects on PIN polarities in the root meristem.

Next we tested the importance of different properties of the model for the auxin effect on PIN polarity. When we removed the auxin-mediated feed-back on PIN transcription from the model causing the reduction of intracellular PIN pool available for recycling, auxin-mediated repolarization was not observed (Fig. S6D-S6I). This largely agrees with previous observations that lines compromised in auxin-mediated transcription show defects in auxin-mediated PIN lateralization (Sauer et al, 2006).

Similarly, when we reduced ABP1 levels in the model with steady state PIN polarity, the auxin-mediated PIN repolarization did not occur (Fig. 5E, 5G), presumably because of reduced PIN internalization rate and decreased intracellular PIN levels. In the contrary, simulations of increased ABP1 levels displayed strong PIN lateralization via enhanced recycling of PINs from preferential polar side and were thus largely insensitive to increased auxin levels in these simulations (Fig. 5C,5F and 5G), features also consistent with experiments using auxin treatment in ABP1 overexpressing lines (Fig. 3C, 3F).

In conclusion, genetic studies and computer model simulations indicate that nuclear and extracellular auxin signalling pathways mediated by distinct auxin receptors are required for auxin effect on PIN polarities. Furthermore, our findings support the crucial role of ABP1 in this auxin effect on PIN protein repolarization.

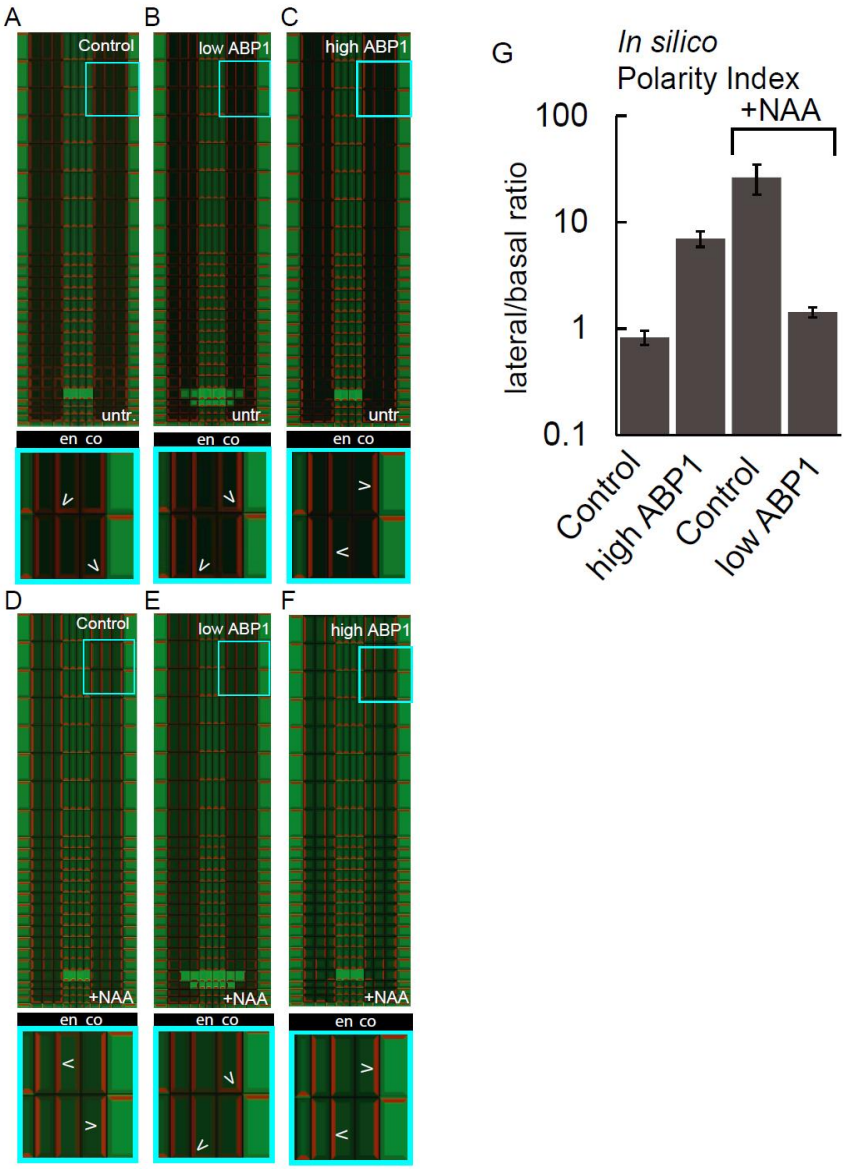


Figure 5. Simulations of auxin-mediated, ABP1-dependent PIN polarizations.
(A-F) Steady-state predictions of PIN polarization from the *in silico* root model without and with ubiquitous auxin production for 'wt-like' situation (A and D), ABP1 reduction (B and E) and ABP1 overexpression (C and F). Panel A-C corresponds to WT [auxin] simulations whereas panels D-F display simulations with *in silico* auxin production.
Bottom panels depict magnification of selected region of the root on the top panels (cyan frame). Abbreviations for root cell types are (en) endodermis; (co) cortex. **(G)** Lateral to basal PIN ratio after virtual auxin treatments predicted by the model for control and ABP1 gain-of-function simulations displayed a similar trend whereas it was compromised in ABP1 loss-of-function mutant simulation. Difference bars depict spatial difference for 10 randomly chosen endodermal/cortex cells along the root axis in the steady state scenario.
Colour coding for model simulations are as in Fig. 2C.

DISCUSSION

Cells in plant tissues are enclosed within a rigid extracellular matrix (the cell wall) therefore tissue polarization and patterning as well as coordinated cell behaviour in general relies on different molecular mechanisms than in animals. Here we identified a central role for the

extracellular auxin receptor ABP1 in auxin-mediated polarization in a mechanism relying on the feedback linking ABP1 to internalization and polarity of PIN auxin transporters.

The genetic manipulation of ABP1 activity in gain- and loss-of-function lines revealed a so far unappreciated role of ABP1 in mediating auxin effect on PIN polarity and hence auxin canalization that is the core of our theoretical model. In particular, our study links auxin perception via ABP1 with the emergence of coordinated cell polarization during developmental processes such organogenesis or vascular tissue formation.

Based on experiments we developed a computer model involving the combination of TIR1/AFB nuclear (Chapman & Estelle, 2009) and ABP1-dependent extracellular auxin sensing (Robert et al, 2010; Xu et al, 2014; Xu et al, 2010) that feeds back on directional auxin movement between cells, which is mediated by polarly localized PIN auxin transporters. The key concept of the model is a common extracellular pool of ABP1 auxin receptors shared between neighbouring cells that is involved in the positive regulation of PIN internalization, thereby translating a global gradient of auxin across tissues into the cascade polarization of individual cells. The model generates coordinated emergence of cell polarities within initially non-polarized tissues closely mimicking the observed pattern during the canalisation processes or within the developing root meristem. Furthermore, the model makes a number of new predictions about auxin canalization and requirements of both nuclear and extracellular auxin-mediated cell polarization opening new venues for future experimentations.

Although various aspects of the proposed mechanism await further investigation, chief among them the behaviour of auxin and auxin receptors in the apoplast, the fair agreement between the experimental results and the outputs of the model simulations support ABP1 as one of the core components of a plant-specific feedback mechanism for the coordination of cell and tissue polarities.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana (L.) Heyhn. seedlings, Columbia ecotype (Col-0), were grown on vertical Murashige and Skoog (0.5 MS) agar plates at 18 °C for 4-5 days.

The transgenic lines used have been described previously: *abp1-5* (Robert et al, 2010); *35S::Calnexin1-GFP (Xu et al, 2014); 35S::ABP1-GFP* with retained KDEL sequence and GFP inserted in the middle of the gene (Robert et al, 2010)*;abp1* knockdown lines (*SS12S*, *SS12K* and *AS*, (Braun et al, 2008)) were induced for 12 or 24 hours before measurements with an 8% ethanol solution kept in a vial at the bottom of vertically oriented plates (Braun et al, 2008).

The *35S::ABP1*; *pRPS5::ABP1-GFP* and *pMDC7_B(XVE)>>ABP1-GFP* plasmids were constructed using the Gateway cloning technology (www.invitrogen.com). The ABP1-GFP coding sequences were transferred from the *pDNR221-ABP1-GFP* entry clone (described in (Robert et al, 2010)) to the gateway-compatible *pMDC7_B(pUBQ10)* destination vector (Barbez et al, 2012) using the Invitrogen LR clonase (+) according to manufacturer's instructions. The ABP1-GFP and the pRPS5 promoter regions were cloned into donor vectors *pDONR221* and *pDONRP4P1R*, respectively. The expression clone was generated by recombining both fragments into the expression vector *pB7m24GW,3*. The resulting constructs were transformed into *Arabidopsis* (Col-0) plants by floral dipping in *Agrobacterium tumefaciens* liquid cultures.

Mutant combinations with *DR5rev::GFP* (Friml et al., 2003), *DII-Venus* (Brunoud et al., 2003) and *PIN1::PIN1-GFP* (Benková et al., 2003) were generated through genetic crosses.

NAA (Sigma-Aldrich) was used from dimethylsulfoxide stock solutions and added to the liquid 0.5 MS growth medium for the indicated times in concentration, if not mentioned otherwise 4h with 10 µM of NAA; In control treatments, equal amounts of solvent were used. For FM4-64 (Invitrogen; in H_2O) accumulation the seedlings were pulse labelled 5 min in MS liquid medium supplemented with 4 µM FM4-64 on ice, washed three times at room temperature in MS liquid medium, mounted and observed immediately.

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SUPPLEMENTARY INFORMATION

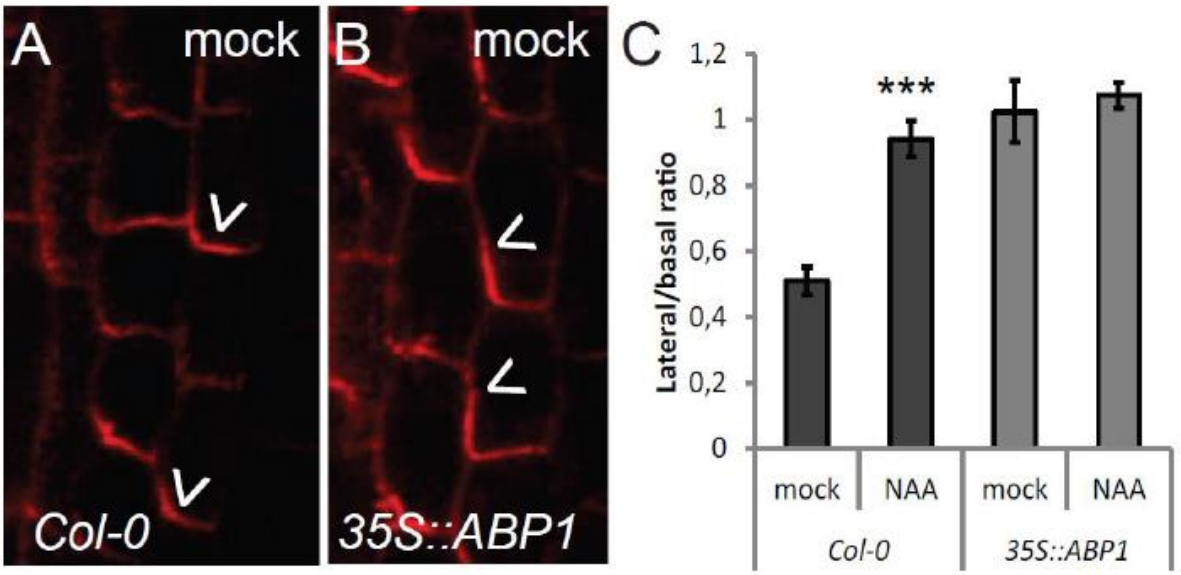

Figure S1. PIN lateralization in an overexpression ABP1 line without GFP.
(A-C) Cellular phenotype of *35S::ABP1* (B) shows PIN1 lateralization in root endodermis cells compared to the WT (A). (C) Quantifications of relative basal-to-lateral PIN1 signal ratio in control and *35S::ABP1*, respectively. Bars indicate standard error. (Student's t-test, ***P < 0.001).

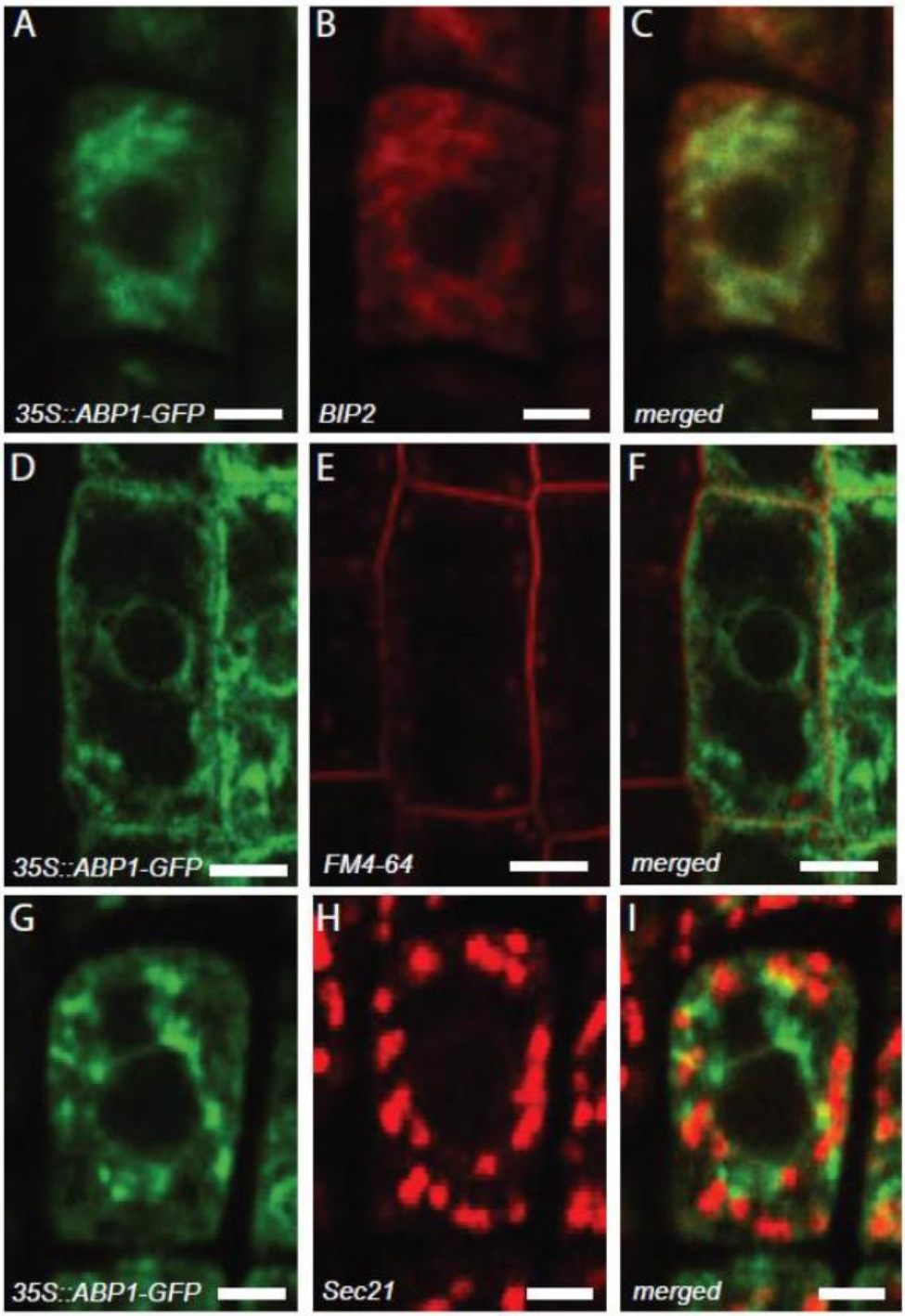

Figure S2. Co-localization of ABP1 with intracellular compartments.
(A-C) Co-localization of ABP1–GFP (green, A) and the ER marker BIP2 (red, B) shown by immunocytochemistry (C). **(D-F)** ABP1–GFP (green, D) does not localize to the plasma membrane (F) (briefly stained by FM4-64, red, E). **(G-I)** No co-localization of ABP1-GFP (green, G) was found with Sec21 (red, H) Golgi marker (I).

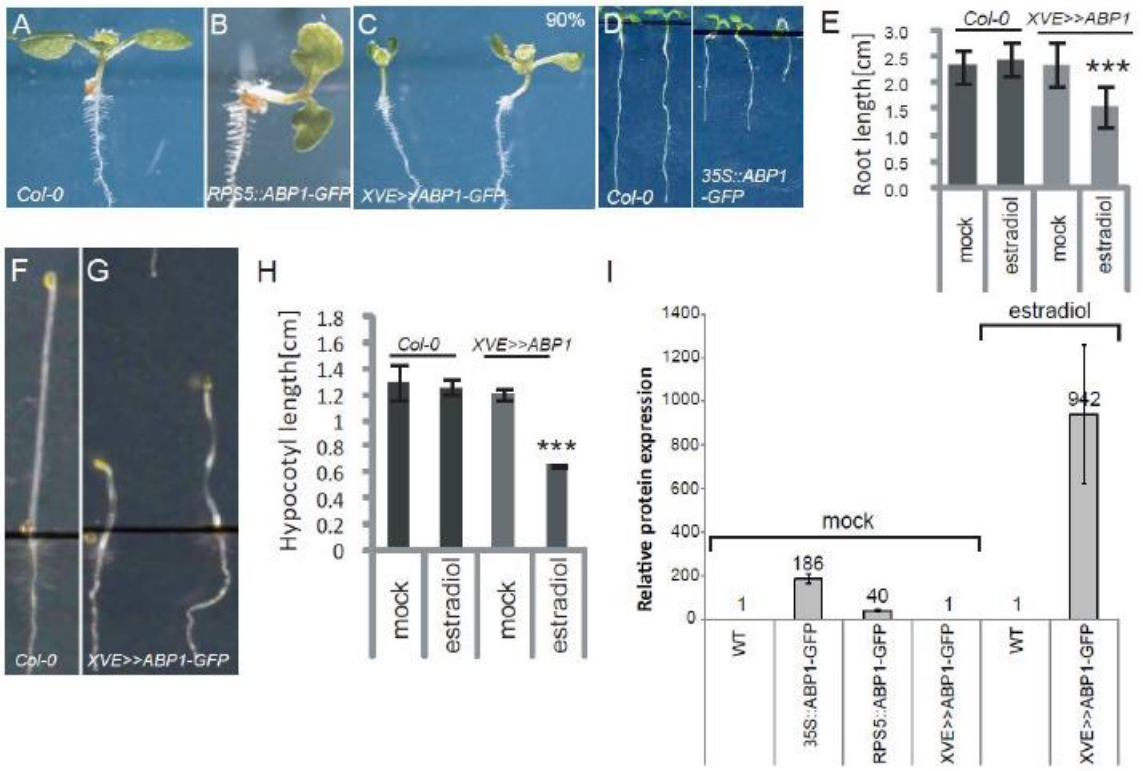

Figure S3. ABP1 role in auxin-mediated seedling development.
(A-H) Phenotypes of vertically grown 7-day-old wild-type, *RPS5::ABP1-GFP, 35S::ABP1-GFP* and *XVE>>ABP1-GFP* ß-estradiol induced seedlings. Mock and induced (2 µM ßestradiol) conditions were compared. Aberrant cotyledons (A-C), reduced primary root growth (D,E) and hypocotyl elongation in four days-days-old seedlings (F-H). (E) Quantification of root length of ABP1-related mutants. Values are the average of three biological replicates (n > 20 on each replicate). Error bars are SEs (Student's t-test, *P < 0.1, ***P < 0.001). (H) Quantification of hypocotyl length of ABP1-related mutants. Values are the average of three biological replicates (n > 20 on each replicate). Error bars are SEs (Student's t-test, *P < 0.1, ***P < 0.001). **(I)** RNA abundance of ABP1 in 10-days-old *wild-type* (*WT*); *35S::ABP1-GFP*; *RPS5::ABP1-GFP*; *XVE>>ABP1-GFP* on mock and germinated on 2 µM estradiol. RNA abundance was determined by qRT–PCR (data are means ± s.d., n > 3).

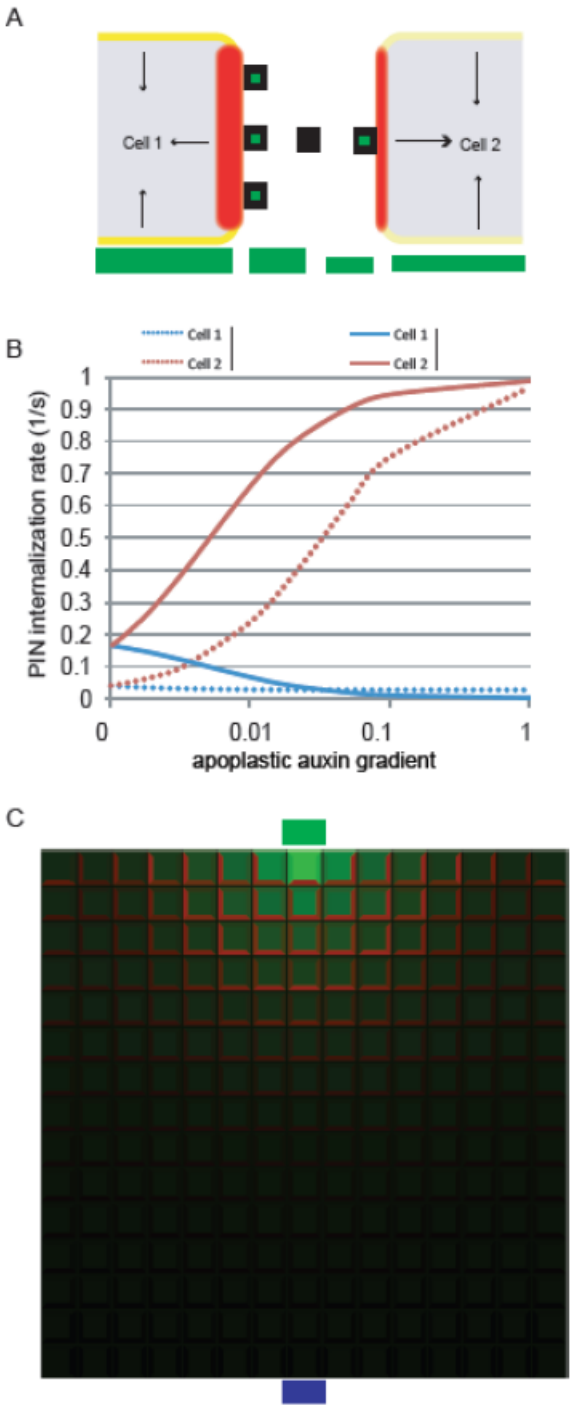

Figure S4. Comparison of Extracellular 'ABP1' model to previously proposed concept **(1).** **(A)** Schematics of model adapter from Wabnik et al.,(1). Symbols are as in Fig. 2A. **(B)** The effective PIN internalization rates of adjacent cells in the function of auxin flux within the cell wall that were predicted by model adapted from Wabnik et al., 2010(1) (a) (dash lines) and the extracellular ABP1 model (Fig. 2A) (solid lines). Note, the extracellular ABP1 model requires smaller auxin gradient to generate significant differences in Pin internalization rates of adjacent cells. **(C)** Simulation of the extracellular ABP1 model without the assumption of fast membrane diffusion of auixn influx carriers. Note impaired PIN polarization in adjacent cells. The auxin source (green horizontal bar) produced auxin at a rate of 0.002 μMμm-2 s-1. The auxin sink (blue horizontal bar) preserves near zero auxin concentrations. Color coding for auxin and PIN levels is as in Fig. 2C.

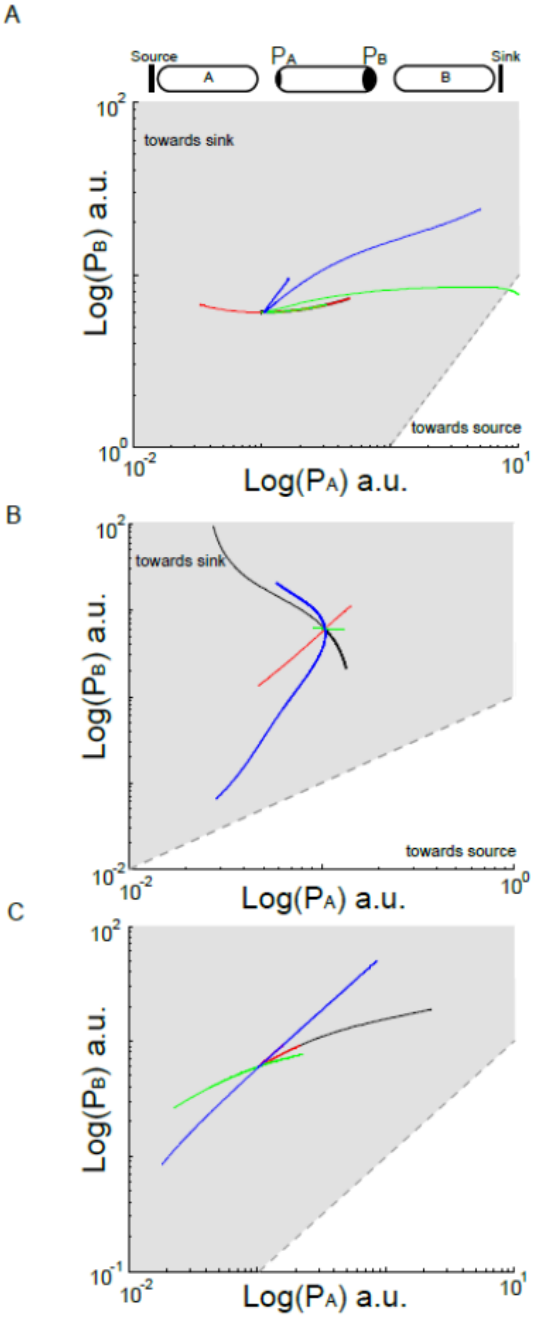

Figure S5. Robustness and sensitivity of the extracellular ABP1 model towards parameter perturbations demonstrated by bifurcation analysis.
(A) Predicted PIN abundance at the side facing cell B is denoted as P_B, and PIN polarization towards cell A (P_A). Small perturbations of model parameters around their nominal rates (+/- 2 order of magnitude) display the robust model output (polarized states) preferentially towards the cell B. The colors of the curves correspond to the parameters: D_C (black), D_R (red), K_D (green) and R_T (blue). Note that large amount of ABP1 leads to weaker polarization is consistent with the experimentally observed lateralization of PIN proteins in ABP1-overexpressing mutants (see Fig. 3). **(B)** Predicted PIN abundance at the side facing cell B is denoted as P_B, and PIN polarization towards cell A (P_A). Small perturbations of model parameters around their nominal rates (+/- 2 orders of magnitude) display robustness of the model output (polarized states) towards the cell B. The colors of the curves correspond to the parameters: D_A (black curve, sensitive parameter, high values led to weak polarities), p_{AUX} (red), p_{IAAH} (green) and

auxin production term (source) (blue). **(C)** Predicted PIN abundance at the side facing cell B is denoted as P_B, and PIN polarization towards cell A (P_A). Small perturbations of model parameters around their nominal rates (+/- 2 order of magnitude) display the prediction output (polarized states) towards the cell B, regardless of parameter choice. The colors of the curves correspond to the parameters: δ_{PIN} (black), k_{endo} (red), k_t (green) and p_{PIN} (blue). The auxin source (denoted as Source) produced auxin at a rate of 0.002 μMμm^{-2} s^{-1}. The auxin sink (denoted as Sink) preserves zero auxin concentrations.

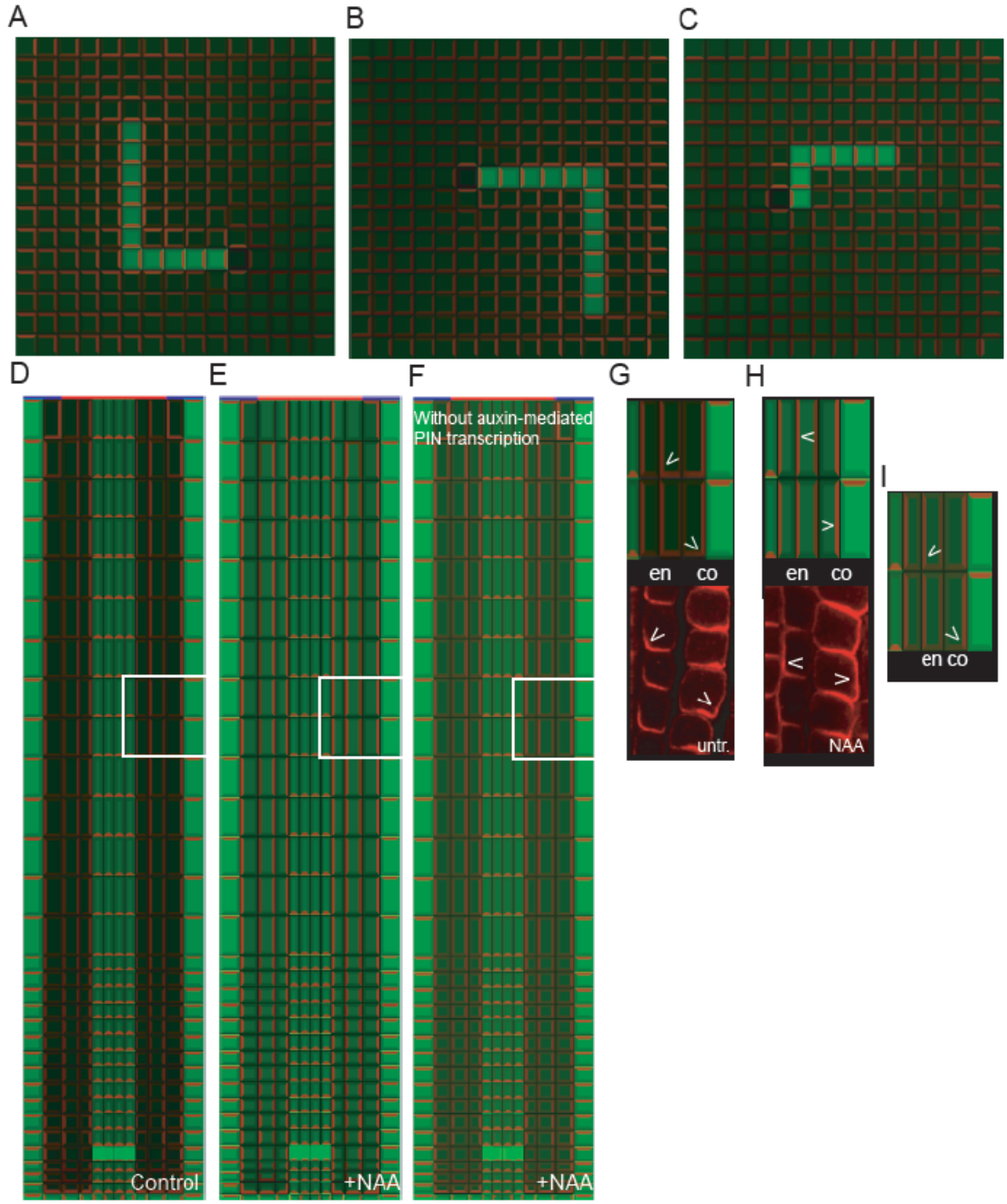

Figure S6. Robustness and dynamics of computer model with respect to locations of auxin production/degradation and lack of auxin-mediated PIN expression.
(A-C) Computer model simulations of the auxin canalization. Model predictions were robust with respect to the particular choice of auxin production (auxin source) or auxin degradation (auxin sink) positions. **(D-F)** Virtual root model simulations of PIN polar rearrangements in

wild-type like scenario (D), virtual auxin applications (E) and when the assumption of auxinmediated PIN expression was removed from the model definition (F**). (G-I)** Comparison of computer model simulations with the *in planta* observations. Wild-type scenario shown without (G), with auxin applications (H) and with auxin-independent PIN expression (I)(2). The auxin source (red bar) produced auxin at a rate of 0.002 $\mu M \mu m^{-2} s^{-1}$. The auxin sink (blue bar) preserves near zero auxin concentrations. Color coding for auxin and PIN levels is as in Fig. 2C.

EXPERIMENTAL PROCEDURES

Transmission Electron Microscopy (TEM)

High-pressure freezing and freeze-substitution for immunogold labeling

Root tips of 3-days-old seedlings of *Arabidopsis thaliana* were excised, immersed in 5% (w/v) sucrose in growth medium, frozen immediately in a high-pressure freezing machine (HPM010; Bal-Tec, Balzers, Liechtenstein) and stored in liquid nitrogen until used further. Freeze substitution was carried out in an EM AFS device (Leica Microsystems, Vienna, Austria). Over a period of four days, root tips were freeze-substituted in anhydrous acetone containing 0.25% (v/v) glutaraldehyde and 0.1% (w/v) uranyl acetate as follows: -80°C for 48 h, gradual warming to -60°C over a 10 h period, -60°C for 16 h, gradual warming to -30°C over a 10 h period, and -30°C for 16 h. At -30°C, samples were rinsed 3 times in acetone for 20 min each, and gradually warmed to 4°C over a 4 h period. Samples were rinsed in dry ethanol (4 times 10 min each) and infiltrated in LR-White (#62662; Fluka, Buchs, Switzerland) overnight at room temperature on a shaking platform. Samples were then brought into gelatin capsules, fully filled and tightly capped, and polymerized at 50°C for 24 h. Ultrathin sections (70-80 nm) were sliced with an ultramicrotome (Leica EM UC7), collected on formvar-coated nickel slot grids and processed for immunogold cytochemistry. Sections were incubated in 50 mM glycine in Tris-buffered saline (TBS; 50 mM, 0.9% NaCl, pH 7.4) for 20 min at RT for quenching of free aldehyde groups, followed by incubation in 2% BSA, 10% normal goat serum, and 1% cold water fish-skin gelatin in TBS containing 0.1% Triton X-100 (TBS-T) for 60 min at RT to block nonspecific binding sites. Primary antibodies, anti-ABP1 (Rabbit), were then applied at a concentration of 1:100 in TBS-T containing 2% BSA overnight at 4°C. After rinsing in TBS-T (four times for 10 min each), goat anti-rabbit immunoglobulins coupled to 10-nm gold particles were applied (British BioCell Int., Cardiff, UK) diluted 1:50 in TBS-T containing 2% BSA and 0.05% polyethylene glycol for 90 min at RT. Sections were then rinsed in double-distilled water

and air dried. Specificity of immunolabeling was controlled omitting primary antibodies with following application of the full set of secondary antibodies.

Sections were contrast enhanced by means of 1% (w/v) uranyl acetate in water for 20 min at RT and 2,66% (w/v) lead nitrate in sodium citrate for 5 min at RT. Sections were examined in a TECNAI 10 TEM operated at 80 kV, equipped with a Morada CCD camera (Soft Imaging Systems, Münster, Germany). Whole images were level adjusted, sharpened and cropped in Photoshop (Adobe) without changing any specific feature within.

Plasmolysis

A fresh protoplasting solution was prepared as following: 0.3% Macerozyme (Yakult Farmaceutical Ind. Co., Ltd.), 0.4M D-Mannitol (Sigma-Aldrich), 20 mM MES monohydrate (DuchefaBiochimie) and 20 mMKCl (Merck). We adjusted the pH to 8.2 for stable GFP observation. The solution was firstly warmed up for 10 minutes at 55°C, then cooled down at RT. 10 mM $CaCl_2$ (Sigma-Aldrich) was added before use.

Immunodetection and Microscopy

Immunofluorescence in *Arabidopsis* embryos, roots and leave primordia was performed as described (Sauer et al, 2006). For immunodetection in roots were used 5 days old seedlings. For immunodetection in leaves were used first leaves of 10 days old seedlings. The anti-PIN1 antibody (1:1000) (Benkova et al, 2003); the anti-PIN2 antibody (1:1000) (Abas et al, 2006); anti-Sec21 rabbit (1:800) (Movafeghi et al, 1999); anti-GFP mouse (Roche; 1:600) and anti-BIP2 (Hsc70), 1:200 (Stressgen Bioreagents) were used, and the fluorochrome-conjugated secondary antibodies the anti-rabbit-Cy3 (1:600) (Dianova); Alexa488 anti-mouse (Invitrogen;1:600); Cy3 anti-mouse,1:600 (Sigma) were used. Live-cell microscopy was done as described (Kleine-Vehn et al, 2008). For FM4-64 dye labeling, 35S::ABP1–GFP seedlings were incubated for 3 min in the medium with a 2μM of FM4-64 dye (Invitrogen) to stain the plasma membrane.

For confocal microscopy, an Exciter confocal scanning microscope (Zeiss, http://www.zeiss.com) was used. Images were processed in Adobe PHOTOSHOP. Each experiment was performed at least three times.

Histological Analysis

For lateral root observation, five-day-old plants were transferred to the plate with 10 µM concentration of NAA (Duchefa) for 60 hours. Consequently roots were cleared based on protocol (Benkova et al, 2003). To observe the cotyledon vascular patterns, 8-10-day-old seedlings were cleared in 30% glycerol containing 2.5 g ml chloral hydrate.

Western blot

Arabidopsis cell suspension culture (PSB-D) were transformed by *Agrobacterium* containing respective construct (ABP1-GSTag, SKU5-GFP, PIP5K-GFP) and cultivated as described in (Van Leene et al, 2007). Cultures with high transgene expression were gradually scaled up to 500 ml. Cell material was separated from media and secreted proteins were extracted by TCA-acetone precipitation (10% TCA in acetone containing 0.07% DTT) (Curto et al, 2006). The final pellet was suspended in 100 µL protein solubilization buffer (9M urea, 4% CHAPS, 0.5% TritonX100, 100 mM DTT) (Maldonado et al, 2008). Presence of secreted protein was detected by Western blot as described in (Eloy et al, 2012).

Quantification of PIN lateralization

Auxin-induced PIN1-lateralization was analysed as described (Sauer et al, 2006). Treatments of five-days-old *Arabidopsis* seedlings with auxin was done for 4 hours in liquid growth medium containing 10 µM NAA (Duchefa) at room temperature and continuous light and were used for anti-PIN1 immunodetection. Endodermis cells from same root region were compared since no difference in meristem size was observed in *abp1* loss- and gain-of-function lines compared with wild type (data not shown). The mean fluorescence intensity of PIN1 signal at the inner lateral and the basal membrane of endodermis cells was measured using ImageJ software (http://imagej.nih.gov/ij/). The quotients between lateral and basal value were calculated for >10 cells of one root and averaged. The averages of at least 10 roots per treatment were subsequently averaged again. P values are from a two-tailed t-test assuming unequal variances. Error bars in all graphs indicate standard error.

Quantitative RT–PCR

Total RNA was extracted with the RNeasy kit (QIAGEN). Poly(dT) cDNA was prepared from total RNA with Superscript III (Invitrogen). Quantitative RT-PCR was done with LightCycler

480 SYBR Green I Master reagents (Roche Diagnostics) and a LightCycler 480 Real-Time PCR System (Roche Diagnostics). Data were analyzed with qBASE v1.3.4 (Hellemans et al, 2007). Expression levels were normalized to the non-auxin-responsive genes β-TUBULIN (At5g12250), EEF (At1g30230) and CDKA (At3g48750).

Following reverse transcription PCR primers sequences were used:

ABP1 Forward primer_1	TCGTCGTCTTTTCCGTCGCG
ABP1 Reverse primer_1	TTGGCAAGCCATTGATGGGACA
ABP1 Forward primer_2	TCCTTGTCCCATCAATGGCTTGC
ABP1 Reverse primer_2	TGCAATACGGAGCCAGCAACAG
CDKA- Forward primer	ATTGCGTATTGCCACTCTCATAGG
CDKA- Reverse primer	TCCTGACAGGGATACCGAATGC
EEF1A4- Forward primer	CTGGAGGTTTTGAGGCTGGTAT
EEF1A4- Reverse primer	CCAAGGGTGAAAGCAAGAAGA
TUB-2_ Forward primer	ACTCGTTGGGAGGAGGAACT
TUB-2_ Reverse primer	ACACCAGACATAGTAGCAGAAATCAAG

COMPUTATIONAL PROCEDURES

Auxin transport dynamics

The auxin flow between the cells is modeled as previously described (Wabnik et al, 2010). In the cytoplasm (pH between 7.2-7.6) auxin is almost completely de-protonated (anionic) and requires proton-driven polar transport that is largely mediated by PIN proteins and well as the family of ABCB transporters (Henrichs et al, 2012; Titapiwatanakun et al, 2009). Here, we primarily focused on PIN proteins as they show asymmetric subcellular localization in the cells that determines the direction of auxin flow (Wiśniewska et al, 2006). The capacity (speed) of PIN-mediated auxin transport is determined by PIN membrane permeability p_{PIN}. Typical values of p_{PIN} used *in planta* can be found in recent report (Kramer et al, 2011). Based on this report we used average value of ~10 μms^{-1}. Detailed analysis of transport parameters of a prototype model can be found in our previous studies (Wabnik et al, 2010; Wabnik et al, 2011). Alternatively, in the acidic cell wall (pH ~ 5.5), fractions of protonated and ionic auxin can either enter the cell via passive diffusion that is described by passive permeability p_{IAAH} or with the help of auxin influx carriers (AUX/LAX) (Swarup et al, 2005) (with permeability p_{AUX}). Additionally, our model Account for the diffusion of auxin across the cell wall determined by diffusion coefficient D_a and corresponding cell wall compartments (Wabnik et al, 2010). Each cell wall compartment (*ij*) is considered to have three neighbors, left and right neighbors (*il, ik*) connected to the same cell i and one neighbor (*ji*) "connected" to the adjacent cell *j* as previosly described(Wabnik et al, 2010). The crossing area between neighboring cytoplasm and membrane/wall compartments (for passive transport) is denoted as l_{ij}, crossing areas between neighboring wall compartments is a_{ijji}, a_{ijik}, a_{ijil} and distances between neighboring wall compartments used in the diffusion terms are given by d_{ijji}, d_{ijik}, d_{ijil}. $a_{ijji} \sim l_{ij}^2$ and *dijji* was 0.1 μm (typical cell wall thickness of 200 nm) whereas for transversal auxin diffusion in the wall we calculated $a_{ijik} = a_{ijil} = (2 \times 0.1^2)^{0.5} \times l_{ij}$. We assumed in our model that auxin binding (association) to the ABP1 protein presumably constrains auxin diffusion in the cell wall. We tested the highest D_a of 5.5 $\mu m^2 s^{-1}$ for which model predictions are robust. This finding was further supported by sensitivity analysis (Fig. S4B) that shows D_a is as sensitive parameter. Used value of apoplastic diffusion was roughly an order of magnitude off the measured diffusion rate for fluorescent dye with similar size to auxin (Kramer et al, 2007). Whether the direct auxin diffusion measurements remains unexplored, we speculate that the reduced auxin diffusion rate in the apoplast reported herein could result from the effective binding of ligand to the signaling components (extracellular receptors or cell wall components) or presumable compactness and heterogeneity of the cell wall primarly affecting diffusibility of chemical

substances. Lower values (up to three orders of magnitude) that one used in this study were previously reported (Bayliss et al, 1996; Canny & Huang, 1994) and used in the recent models of plant development (Ibanes et al, 2009).

Dynamics of auxin transport is given by:

$$\frac{dIAA_i}{dt} = \frac{1}{V_i} \cdot \left[p_{IAAH} \cdot \sum_{j \in N_i} l_{ij} \cdot (f_{in}^{+}(IAA_{ij}) - f_{out}^{+}(IAA_i)) \right]$$

$$+ \quad \frac{1}{V_i} \cdot \left[p_{PIN} \cdot \sum_{j \in N_i} PIN_{ij} \cdot (f_{in}^{-}(IAA_{ij}) - f_{out}^{-}(IAA_i)) \right]$$

(1)

$$+ \quad \frac{1}{V_i} \cdot \left[p_{AUX} \cdot \sum_{j \in N_i} AUX_{ij} \cdot (f_{out}^{-}(IAA_{ij}) - f_{in}^{-}(IAA_i)) \right]$$

$$\frac{dIAA_{ij}}{dt} = \frac{1}{V_{ij}} \cdot \left[D_a \cdot \left\{ \frac{a_{ijji}}{d_{ijji}} \cdot (IAA_{ji} - IAA_{ij}) + \frac{a_{ijik}}{d_{ijik}} \cdot (IAA_{ik} - IAA_{ij}) + \frac{a_{ijil}}{d_{ijil}} \cdot (IAA_{il} - IAA_{ij}) \right\} \right]$$

$$+ \quad \frac{1}{V_{ij}} \cdot \left[p_{IAAH} \cdot l_{ij} \cdot (f_{out}^{+}(IAA_i) - f_{in}^{+}(IAA_{ij})) \right]$$

$$+ \quad \frac{1}{V_{ij}} \cdot \left[p_{PIN} \cdot PIN_{ij} \cdot (f_{out}^{-}(IAA_i) - f_{in}^{-}(IAA_{ij})) \right]$$

(2)

$$+ \quad \frac{1}{V_{ij}} \cdot \left[p_{AUX} \cdot AUX_{ij} \cdot (f_{in}^{-}(IAA_i) - f_{out}^{-}(IAA_{ij})) \right]$$

with

$$f_{in}^{+}(IAA_{ij}) = \frac{IAA_{ij}}{1+10^{pH_{wall}-pK}}, \quad f_{out}^{+}(IAA_{i}) = \frac{IAA_{i}}{1+10^{pH_{cell}-pK}},$$

$$f_{in}^{-}(IAA_{ij}) = \frac{\Phi_{influx}}{1+10^{-pH_{wall}+pK}} \cdot \frac{IAA_{ij}}{k_t + IAA_{ij}}, \quad f_{in}^{-}(IAA_{i}) = \frac{\Phi_{influx}}{1+10^{-pH_{cell}+pK}} \cdot \frac{IAA_{i}}{k_t + IAA_{i}},$$

$$f_{out}^{-}(IAA_{ij}) = \frac{\Phi_{efflux}}{1+10^{-pH_{wall}+pK}} \cdot \frac{IAA_{ij}}{k_t + IAA_{ij}}, \quad f_{out}^{-}(IAA_{i}) = \frac{\Phi_{efflux}}{1+10^{-pH_{cell}+pK}} \cdot \frac{IAA_{i}}{k_t + IAA_{i}}$$

(3)

where IAA_i is the mean auxin concentration in the *i*-th cell and IAA_{ij}, IAA_{ji}, IAA_{ik}, IAA_{il}, are the mean auxin concentrations in adjacent wall compartments (Fig. 2A, main text), V_i and V_{ij} are the dimensions of the cell and wall compartment, respectively. N_i denotes the number of direct neighbors of cell *i*. The PIN_{ij} and AUX_{ij} variables determine the average level of PINs and AUX/LAXs carriers at the *i*-th plasma membrane facing cell *j*. The parameter k_t defines the saturation constant of polar auxin transport. D_a describes auxin diffusion between the neighboring wall compartments. p_{IAAH}, p_{PIN}, p_{AUX} are the membrane permeabilities for passive diffusion and carrier mediated transport, respectively. The pH differs between cytoplasm and extracellular space (pH_{cell}, pH_{wall}) leading to different auxin fractions inside/outside of the cell: $f_{in}^{+}(IAA_{ij})$, $f_{in}^{-}(IAA_{ij})$, $f_{in}^{-}(IAA_i)$, $f_{out}^{+}(IAA_i)$, $f_{out}^{-}(IAA_{ij})$, $f_{out}^{-}(IAA_i)$. Each wall compartment (*ij*) is considered to have three neighbors, left and right neighbors (*il*, *ik*) connected to the same cell *i* and one neighbor (*ji*) "connected" to the neighboring cell *j*. The crossing area between neighboring cytoplasm and membrane/wall compartments is denoted as l_{ij}, crossing areas between neighboring wall compartments is a_{ijji}, a_{ijik}, a_{ijil} and distances between neighboring wall compartments used in the diffusion terms are given by d_{ijji}, d_{ijik}, d_{ijil}. The active auxin transport mediated by PINs and AUX/LAXs proteins also depends on the electrochemical gradient between cytoplasm and the apoplast. The Φ_{influx} and Φ_{efflux} parameters (eq. 3) describe the membrane potential:

$$\Phi_{influx} = \Phi_{efflux} \cdot e^{\frac{zVF}{RT}} = \frac{zVF}{RT} \cdot \frac{e^{\frac{zVF}{RT}}}{e^{\frac{zVF}{RT}} - 1},$$

(4)

where $V = -100\ mV$, $F = 9.6 \times 10^4\ mol^{-1}$, $R = 8.3\ Jmol^{-1}K^{-1}$, $T = 300K$.

Recycling and lateral diffusion of PIN and AUX/LAX proteins

PIN and AUX/LAX proteins recycle between endosomes and plasma membrane with the base rates a_{exo}, k_{exo} and a_{endo}, k_{endo} for AUX/LAX and PIN exocytosis (trafficking from endosomes to the plasma membrane) and their internalization (trafficking from plasma membrane to the endosomes), respectively. AUX/LAX transporters are distributed evenly on the cell membrane since they are largely considered non-polar subcellular localization.

The AUX/LAX carriers are allocated in the plasma membrane in each time step as follows:

$$\frac{dAUX_{ij}}{dt} = a_{exo} \cdot AUX_i - a_{endo} \cdot AUX_{ij} \cdot (1 + ABP1_{ij}^{QSS}) + D_{AUX} \cdot \sum_{k \in N_i} (AUX_{ik} - AUX_{ij})$$

(5)

where AUX_{ij} represents the average amount of AUX/LAX proteins at the plasma membrane, and AUX_i is a total intracellular level of AUX/LAX in cell i and a_{exo} and a_{endo} are the rates of AUX/LAX exocytosis and internalization, respectively. D_{AUX} is AUX/LAX lateral diffusion between pair of adjacent plasma membrane compartments. $ABP1_{ij}$ determines the amount of free ABP1 at the surface of i-th cell facing the adjacent j-th cell.

The corresponding intracellular AUX/LAX levels in *ith* cell are described as follows:

$$\frac{dAUX_i}{dt} = \sum_{j \in N_i} (a_{endo} \cdot AUX_{ij} \cdot (1 + ABP1_{ij}^{QSS}) - a_{exo} \cdot AUX_i)$$

(6)

The polar, subcellular localization of PIN auxin efflux facilitators in the model is determined by differential PIN retention at a given cell side as a result of an auxin-dependent inhibition of PIN internalization through ABP1 protein (Paciorek et al, 2005; Robert et al, 2010).

PIN allocation in the plasma membrane changes according to the following formula:

$$\frac{dPIN_{ij}}{dt} = k_{exo} \cdot PIN_i - PIN_{ij} \cdot k_{endo} \cdot (1 + ABP1_{ij}^{QSS}) + D_{PIN} \cdot \sum_{k \in N_i} (PIN_{ik} - PIN_{ij})$$

(7)

where PIN_{ij} are the PIN level on *ij*-th plasma membrane, and PIN_i is the total intracellular PIN level in *i*-th cell. The parameter k_{exo} determines the rate of PIN exocytosis, and k_{endo} is a base rate for PIN endocytosis whereas $ABP1_{ij}$ determines the amount of free ABP1 at the surface of *i*-th cell facing the adjacent *j*-th cell. D_{PIN} is a slow PIN lateral diffusion (~0.01 μms^{-1}) ($D_{AUX} >> D_{PIN} \sim 1\ \mu ms^{-1}$) between pair of adjacent plasma membrane compartments. Based on experimental data, we assumed that auxin influx proteins undergo faster lateral diffusion than PIN efflux transporters (Kleine-Vehn et al, 2011).

The corresponding dynamics of intracellular PIN level in *ith* cell is given by:

$$\frac{dPIN_i}{dt} = \sum_{j \in N_i} (PIN_{ij} \cdot k_{endo} \cdot (1 + ABP1_{ij}^{QSS}) - k_{exo} \cdot PIN_i)$$

(8)

PIN and AUX/LAX expression

We model the expression of AUX/LAX and PIN proteins in the cell as follows:

$$\frac{dPIN_i}{dt} = \alpha_{PIN} \cdot \frac{IAA_i}{k_m + IAA_i} - \delta_{PIN} \cdot PIN_i$$

(9)

$$\frac{dAUX_i}{dt} = \alpha_{AUX} \cdot \frac{IAA_i}{k_m + IAA_i} - \delta_{AUX} \cdot AUX_i$$

(10)

where PIN_i and AUX_i are the total intracellular concentrations of PIN and AUX/LAX in cell *i*, α_{PIN} and α_{AUX} define the rates of auxin-induced PIN and AUX/LAX synthesis and δ_{PIN} and δ_{AUX} determine decay rates of PIN and AUX/LAX proteins. IAA_i expresses the mean auxin concentration in the *i*-th cell and k_m is a Michaelis–Menten constant for auxin-dependent PIN and AUX/LAX expression.

Quasi-steady state ABP1 dynamics in the cell wall

Our model assumes that two adjacent cells contributes to ABP1 secretion to the common pool in the cell wall denoted as $2*ABP1_T$. This pool of free ABP1 proteins positively acts on PIN protein internalization (Robert et al, 2010) on the surface of the cell. Auxin binds ABP1 in the cell wall and facilitates the formation of inactive auxin-ABP1 complex (occupied receptor) that can freely diffuse within the cell wall. The amount of ABP1 remains conserved in the cell wall thereby the mass conservation law can be written:

$$2 \cdot ABP1_T = ABP1_{ij} + ABP1_{ji} + ABP1^*_{ij} + ABP1^*_{ji}$$

(11)

where $ABP1_{ij}$ and $ABP1_{ji}$ are free ABP1 on the surface of *i*-th cell and *j*-th cell, respectively and $ABP1_{ij}^*$ and $ABP1_{ji}^*$ are corresponding auxin-bound ABP1 complexes. The kinetics of the reversible auxin-ABP1 binding is given by:

$$IAA_{ij} + ABP1_{ij} \xleftrightarrow{k_f, k_r} ABP1^*_{ij}, \quad IAA_{ji} + ABP1_{ji} \xleftrightarrow{k_f, k_r} ABP1^*_{ji}$$

(12)

where k_f and k_r are forward and backward rates of ABP1 association and dissociation states, respectively. A dissociation constant of auxin-ABP1 complex (K_D) is determined as:

$$K_D = \frac{kr}{kf}$$

(13)

The relative changes in the amount of auxin-bound and free ABP1 are governed by following ODE system:

$$\frac{dABP1^*_{ij}}{dt} = IAA_{ij} \cdot ABP1_{ij} - K_D \cdot ABP1^*_{ij} + D_c \cdot (ABP1^*_{ji} - ABP1^*_{ij})$$

(14)

$$\frac{dABP1^*_{ji}}{dt} = IAA_{ji} \cdot ABP1_{ji} - K_D \cdot ABP1^*_{ji} + D_c \cdot (ABP1^*_{ij} - ABP1^*_{ji})$$

(15)

$$\frac{dABP1_{ij}}{dt} = -IAA_{ij} \cdot ABP1_{ij} + K_D \cdot ABP1^*_{ij} + D_R \cdot (ABP1_{ji} - ABP1_{ij})$$

(16)

$$\frac{dABP1_{ji}}{dt} = -IAA_{ji} \cdot ABP1_{ji} + K_D \cdot ABP1^*_{ji} - D_R \cdot (ABP1_{ji} - ABP1_{ij})$$

(17)

where D_R is a free ABP1 diffusion coefficient and D_C denotes the diffusion of the auxin-ABP1 complex. We found that the assumption a quasi-steady state levels of auxin-ABP1 complexes and free ABP1 is a fairly good approximation of full ODE system dynamics. Given that the ABP1 pool per each pair of adjacent cells is conserved we assume that the transversal diffusion of receptors can be negligible. One can assign the right side of equations (14)-(16) to zero whereas equation (17) can be replaced by equation (11). By solving the linear system of

equations (11), (14)-(16) one obtains the following relations for the amounts of free ABP1 acting on PIN internalization:

$$ABP1_{ij}^{QSS} = \frac{2 \cdot ABP1_T \cdot K_D (\Delta \cdot K_D + 2 \cdot \sqrt{\Delta \cdot \Phi} + IAA_{ji})}{\Delta \cdot K_D \cdot (2 \cdot K_D + IAA_{ij} + IAA_{ji}) + 2 \cdot \sqrt{\Delta \cdot \Phi} \cdot (IAA_{ij} + IAA_{ji} + 2 \cdot K_D) + (2 \cdot IAA_{ij} \cdot IAA_{ji} + IAA_{ij} \cdot K_D + IAA_{ji} \cdot K_D)}$$

(18)

$$ABP1_{ji}^{QSS} = \frac{2 \cdot ABP1_T \cdot K_D (\Delta \cdot K_D + 2 \cdot \sqrt{\Delta \cdot \Phi} + IAA_{ij})}{\Delta \cdot K_D \cdot (2 \cdot K_D + IAA_{ij} + IAA_{ji}) + 2 \cdot \sqrt{\Delta \cdot \Phi} \cdot (IAA_{ij} + IAA_{ji} + 2 \cdot K_D) + (2 \cdot IAA_{ij} \cdot IAA_{ji} + IAA_{ij} \cdot K_D + IAA_{ji} \cdot K_D)}$$

(19)

where $\Delta = \frac{D_R}{D_C}$, and $\Phi = D_C \cdot D_R$.

The free ABP1 associates on the surface of a given cell (Fig. 2A). Given that free ABP1 modulate locally clathrin-dependent endocytosis of plasma membrane proteins (Robert et al, 2010) they would require to be retained at the cell surface (represented by discrete wall compartment). To account for this possibility, we assume that the diffusion of free ABP1 (signaling unit) is compromised and much slower than the diffusion of auxin-bound ABP1 ($D_R << D_C$).

Numerical and simulation methods

The cellular tissue templates for model simulations were created with the version of VV (Vertex-Vertex) programming language (Prusinkiewicz & Runions, 2012) and in the L-system-based modeling software L-studio (http://algorithmicbotany.org/lstudio). The model simulations were done by numerical computations of coupled ODE systems, with an adaptive-size, implicit Cranck Nicholson method with monitoring of local truncation error to ensure accuracy and adjustment of the step size. A maximal time interval of 1 s was used, but other values were also tested without significant changes in the qualitative results of the simulations.

For the sensitivity and bifurcation analysis of the stationary solutions (Fig. S4), we used MATCONT - graphical Matlab package for numerical bifurcation analysis (Dhooge et al, 2003).

Boundary conditions

In the computer model, each cell is abstracted by two subcellular compartments, the cytoplasm compartment and the plasma membrane compartment (Fig. 2A). The plasma membrane is divided into four subcomponents, each being associated with a given side of the cell. To account for a realistic root tissue layout, the cell length varies along the main root axis (50 cells of 60 µm length, 120 cells of 30 µm, 50 cells of 20 µm) as observed *in planta*. Also differences in the cell width are incorporated: 30 µm for root epidermis and QC cells, 25 µm for cortex and endodermis cells, and 15 µm for vascular cells (Fig. 2E, F). Model simulations of auxin canalization (Fig. 2D, Fig. S3C) and auxin and PIN distribution in the root (Figs. 2E-2G and 3A-3F, the auxin source (shown as green horizontal bars in canalization and root simulations) produced auxin at a rate of 0.002 µMµm^{-2} s^{-1}. The auxin sink (shown as blue bar) was a place where auxin was evacuated from the system (sink preserves zero auxin concentration). For the remaining tissue borders in all model simulations, zero-flux boundary conditions were used. In simulations of auxin applications presented in Fig. 3D-3F and Fig. S5E-S5I, each cell of the virtual root produced auxin at the rate of 0.0001 µMµm^{-2}.

Summary of model parameters

Parameter	Value	Units	Reference
Apoplastic diffusion (D_a)	5 (default); +/- 2 orders of magnitude perturbations are shown in Fig. S4B (sensitive parameter)	µm^2 s^{-1}	This study and (Ibanes et al, 2009;

			Kramer et al, 2007)
Constrained ABP1 diffusion in aggregates (D_R)	0.0001(default); +/- 2 orders of magnitude perturbations are shown in Fig. S4A	$\mu m^2\ s^{-1}$	This study
Auxin-receptor complex diffusion (D_C)	1 (default); +/- 2 orders of magnitude perturbations are shown in Fig. S4A	$\mu m^2\ s^{-1}$	This study
IAAH permeability (p_{IAAH})	10 (default); +/- 2 orders of magnitude perturbations are shown in Fig. S4B	$\mu m\ s^{-1}$	(Goldsmith et al, 1981; Swarup et al, 2005)
PIN permeability (p_{PIN})	20 (default); +/- 2 orders of magnitude perturbations are shown in Fig. S4C	$\mu m\ s^{-1}$	(Kramer et al, 2011)
AUX/LAX permeability (p_{AUX})	20(default); +/- 2 orders of magnitude perturbations are shown in Fig. S4B	$\mu m\ s^{-1}$	(Goldsmith et al, 1981; Swarup et al, 2005)
pH in wall (pH_{wall})	5.5	-	(Kramer et al, 2011)
pH in cell (pH_{cell})	7.6	-	(Kramer et al, 2011)
Dissociation constant (pK)	5	-	(Kramer et al, 2011)
Saturation constant for auxin transport (k_t)	1(default); +/- 2 orders of magnitude perturbations are shown in Fig. S4C	µM	(Kramer et al, 2011)
PIN exocytosis base rate (k_{exo})	1(default)	s^{-1}	(Wabnik et al, 2010)
PIN internalization base rate (k_{endo})	0.001 (default); +/- 2 orders of magnitude perturbations are shown in Fig. S4C	s^{-1}	(Wabnik et al, 2010)
AUX/LAX exocytosis base rate (a_{exo})	1(default)	s^{-1}	(Wabnik et al, 2010)
AUX/LAX internalization base rate (a_{endo})	0.001(default)	s^{-1}	(Wabnik et al, 2010)

PIN production rate (α_{PIN})	1(default); 0 (Fig. S5F);	s^{-1}	This study
PIN degradation rate (δ_{PIN})	0.02 (default); +/- 2 orders of magnitude perturbations are shown in Fig. S4C	s^{-1}	This study
AUX/LAX production rate (α_{AUX})	1 (default)	s^{-1}	This study
AUX/LAX degradation rate (δ_{AUX})	0.05 (default)	s^{-1}	This study
Saturation of auxin-induced PIN and AUX/LAX production (k_m)	100 (default)	µM	(Wabnik et al, 2010)
Receptor dissociation constant (K_D)	0.2 (default); +/- 2 orders of magnitude perturbations are shown in Fig. S4A	µM	(Tromas et al, 2010)
The number of extracellular auxin receptors (R_T)	100 (default); 0.1 (Fig. 3B, 3E); 10000 (Fig. 3C, 3F); +/- 2 orders of magnitude perturbations are shown in Fig. S4A	a.u.	This study

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Conclusions and Perspectives

Conclusions and perspectives

As sessile organisms, plants are not able to escape from unfavourable environments like most animals do. Plants need to react to the environmental stimuli to adapt their growth. Shade avoidance is a mechanism that helps plant to compete for light in the dense vegetation, whereas gravitropism and phototropism are responses to environmental changes to enable roots to reach for water and nutrients and shoots to position optimally for light perception.

During this PhD project, the main focus was to gain a better understanding into processes that control cell polarity of auxin transporters PIN-FORMED (PIN) in *Arabidopsis thaliana*. The plant hormone auxin has been described as one of the most important regulators of growth, development and responses to changing environment in plants (Vanneste and Friml, 2009). Auxin plays a central role in plant ontogenesis coordinating developmental events through its differential distribution in the tissues. The establishment of those 'so-called' auxin gradients is formed thanks to the directional transport of the hormone facilitated by the polar localization of its transporters (PINs) in the plasma membrane (PM) (Benková et al., 2003; Friml et al., 2003; Wiśniewska et al, 2006). This organised distribution of PIN is possible due to the concerted action of the subcellular machinery. PIN polarity regulation triggers our interest in further investigating the concepts that underlie PIN polarity establishment in different plant tissues and during various plant developmental stages. Characterisation of this mechanism requires further work focused on the identification of molecular players regulating specifically the trafficking of PINs and is of great importance to understand the regulation of auxin transport.

In the first part of this thesis, we gave an overview of the recent knowledge on plant tropic responses and the role of regulated auxin accumulation during tropisms. We summarized the differential PIN3 PM localization to regulate hypocotyl growth responses to changing environment. The intracellular components that regulate PIN3 polarity are discussed in more detail (**Chapter 1**). Although the plant gravi- and phototropism have been studied extensively, the molecular basis for the PIN3 polarity regulation during those processes is still largely unknown.

Polarization of PIN3-dependent auxin transport for hypocotyl gravitropic response

Previous reports have revealed that PIN3 regulates an auxin maximum redistribution during tropic responses (Ding et al., 2011; Friml et al., 2002). These results prompted us to study the regulation of PIN3 and the impact of this relocation on tropic bending responses. In

Chapter 2 we addressed a mechanism of PIN3-dependent gravitropism of the Arabidopsis hypocotyl. Obtained data present the evidence that PIN3 relocates in endodermal cells after gravity stimulation and regulates auxin accumulation to the lower hypocotyl side. Increased auxin concentration in lower epidermal cells induces cell elongation which then results in hypocotyl bending. We further tested the intracellular regulation of PIN3 gravity-induced relocation (Figure 1a). PIN3 relocation depends on PINOID (PID) kinase-mediated phosphorylation and on ARF-GEF GNOM function. Moreover, we showed that this polarity change is not based on protein biosynthesis or protein degradation (Rakusová et al., 2011; Figure 1a).

We covered only a little of the puzzle, aiming to understand plainly the gravitropic signaling pathway. There are still missing points to be analysed, for instance what is the link between amyloplasts relocation to the bottom of the endodermal cells (Hashiguchi et al., 2013) and PID (de)activation. Furthermore, PIN3 relocation during phototropism is definitely more interesting to study since the signal induced after the light perception probably in non-endodermal cells in the upper part of the plant (Preuten et al., 2013; Liscum et al., 2014) leads ectopically to PIN3 relocation in the endodermis. Taking together, we know that light and gravity perceptions probably occur at different places but converge to a similar output – PIN3 relocation, auxin accumulation resulting in organ bending. A systematic comparison of light and gravity signaling would uncover the convergence point to the two tropic responses.

Auxin feed-back on PIN3 polarity for termination of shoot tropic responses

One of the surprising findings during my PhD study was the identification of a regulatory function of auxin itself on PIN3 in hypocotyl endodermis that might act as one of the check points in regulating gravity-induced bending response. After auxin application on etiolated hypocotyls we observed a rapid decrease of PIN3 protein abundance from the outer PM side in hypocotyl endodermal cells. This process is slower (4 hours) than gravity induced PIN3 relocation (2 hours) and uses different intracellular regulation. Altogether, the data indicate that auxin interferes preferentially with trafficking pathways directing PIN3 from the outer membranes into the vacuole, which leads to a significant extinction of PIN3 specifically from outer cell side, but not from the inner membranes. We describe in more detail in **Chapter 3** the cellular mechanism of this process and we compare it to PIN3 cellular polarity regulation during gravity-induced PIN3 relocation (Figure 1a and 1b).

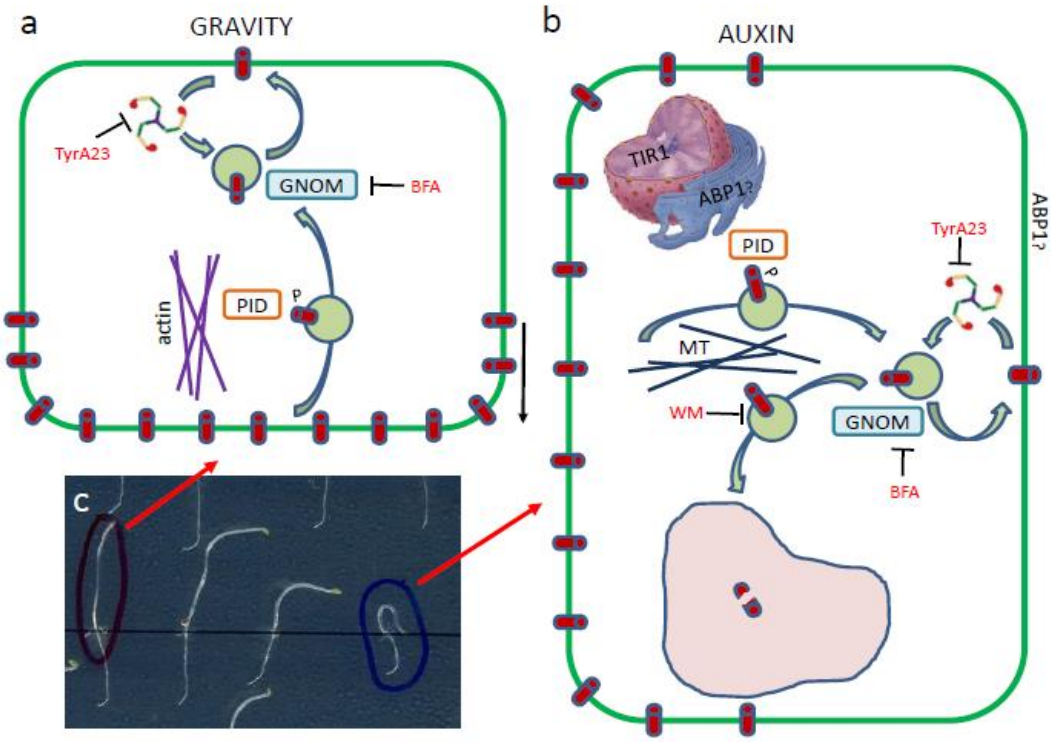

Figure 1. PIN3 polarity regulation during gravitropism and auxin-mediated feed-back regulation of gravitropic over-bending.
(a) Scheme represents PIN3 relocation in hypocotyl endodermal cells after 2 hours of gravity stimulation. Intracellular regulation of PIN3 relocation in hypocotyl endodermal cells relies on clathrin-dependent endocytosis and GNOM-regulated intracellular cycling. PINOID (PID)-mediated phosphorylation play important role in PIN3 polar targeting and presumably also PIN3 activity regulation. Gravity-induced PIN3 relocation is actin dependent.
(b) Scheme represent PIN3 inner-lateralization in hypocotyl endodermal cells after 4 hours of auxin treatment or prolonged gravity stimulation after around 19 hours when auxin accumulated in lower hypocotyl side feed back on PIN3 localization in endodermal cells. Auxin-induced PIN3 inner-lateralization (left represent inner side with still present PIN3 proteins, right represent outer side of hypocotyl endodermal cells) is clathrin and GNOM-dependent. This process is regulated by PID-mediated phosphorylation. The PIN3 regulated degradation, presumably vie vacuolar targeting is the main mechanism. Auxin-mediated PIN3 inner-lateralization is microtubule (MT) dependent. The process is mediated preferentially by Auxin-binding-protein1 (ABP1)-mediated auxin signaling pathway.
(c) Anticipated gravity bending responses when inhibiting gravity-induced PIN3 relocation (agravitropic phenotype; marked red) or auxin-mediated feed-back on PIN3 during regulation of over-bending (over-bending response, marked blue). Arows indicate anticipated phenotype in case of defect during gravity or auxin-mediated PIN3 polarity change.
TyrA23 – tyrphostin 1; PID – PINOID kinase; MT – microtubules; BFA – brefeldin A; ABP1 – auxin binding protein 1.

The molecular mechanism by which auxin regulates PIN3 degradation and most likely reduces auxin transport to the lower hypocotyl side remains elusive and many questions still need to be answered. For instance, does (and how does) the auxin signaling pathway distinguish phosphorylated over non-phosphorylated PIN3 before recruiting it to the degradation pathway? The data presented the role of ABP1 during auxin-mediated PIN3 inner-lateralization does not explain, if the ABP1-auxin signaling is in the endoplasmic reticulum or in the apoplast. This could be tested by using an ABP1 mutated line where ABP1 is not secreted.

To answer these and other questions, targeted forward genetic screens to clarify the process involved in the gravity effect on PIN3 relocation and auxin effect on PIN3 degradation may fill the missing gaps in understanding the mechanism of PIN3 polarity regulation.

Identification of new PIN3 polarity regulators in *Arabidopsis* gravity-stimulated hypocotyl

The obtained results during the first term of my PhD clearly indicate the important role of PIN3 during gravity and light responses and unveiled the importance of auxin feed-back regulation for plant tropism. We demonstrated that PIN3 relocates after gravity stimulation to redirect auxin transport toward the new hypocotyl lower side where auxin-dependent cell elongation results in gravitropic bending. In the second term of the PhD, I followed up the obtained data to have a closer look at the specific processes where PIN3 is involved. Our attempts using forward (Figure 2) and reverse genetic approaches to identify mutants defective in phototropic and gravitropic responses and more specifically in PIN3 localization in the hypocotyl give us the opportunity to characterize these new components in tropic signalling pathways.

The forward genetic screen presented in **Chapter 4** was based on stimulating etiolated seedlings and selecting mutants with an altered hypocotyl bending response (Figure 2b). Mutants with agravitropic phenotypes may carry mutations in genes involved in the regulation of gravity-induced PIN3 relocation. Mutants with over-bending response may exhibit problem in auxin-mediated PIN3 inner-lateralization.

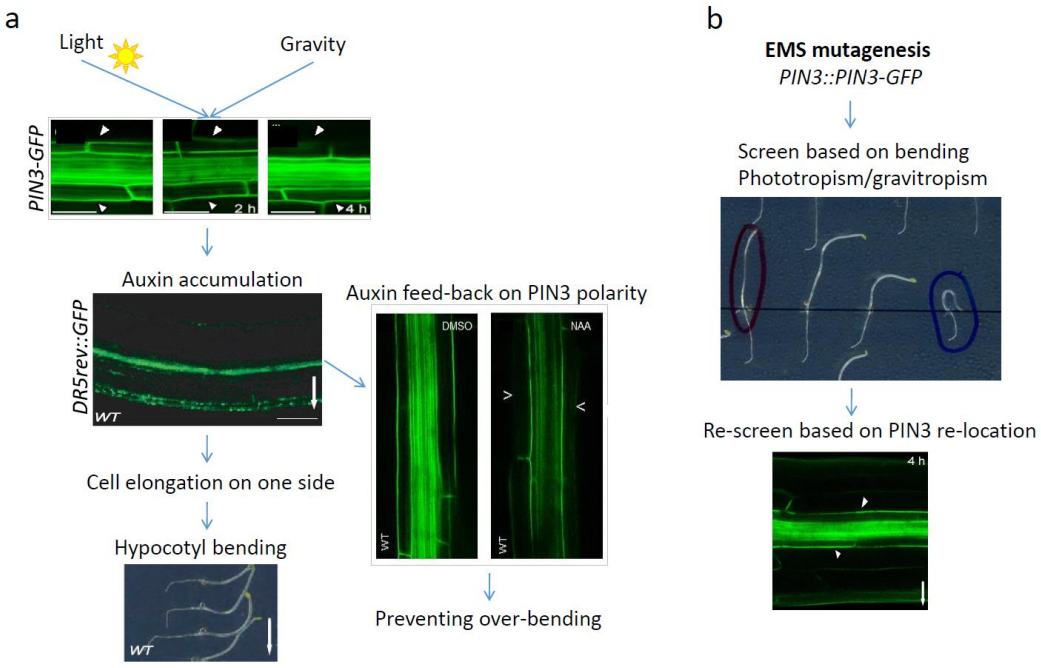

Figure 2. Design of forward genetic screen for regulators of PIN3 polarity in *Arabidopsis* hypocotyls
Here we show the working flow of a novel forward genetic screen for molecular components of PIN3 polarity.

(a) Light and gravity induce PIN3 polarity change, followed by auxin accumulation and cell elongation in lower/shaded hypocotyl side, and finally hypocotyl bending. Besides, auxin feed-back regulates its own accumulation via PIN3 polarity regulation to prevent hypocotyl over-bending.
(b) The screen is based on the evaluation of gravitropic hypocotyl growth and PIN3 polar localization in the transgenic *PIN3::PIN3-GFP* background. The screen has been designed considering the hypothesis that the EMS-induced mutations in putative regulators of PIN3 polarity would prevent changes of PIN3 relocation in the endodermal cells during gravitropism and phototropism. These mutants are expected to not bend (circled in red). The second category of mutants would to be resistent to auxin-induced PIN3 inner-lateralizaton resulting in hypocotyl over-bending after gravity stimulation (circled in blue). Preventing PIN3 relocation after tropic stimulation will subsequently result in the prevention of the auxin flow towards lower side of hypocotyl, no asymmetric auxin distribution in the gravistimulated hypocotyl and no or altered gravitropic response.

So far I have selected a total of 9 candidates from both categories – agravitropic and overbending mutants, which were sent for whole genome sequencing. The best candidates from the list of mutations will be tested. Confirmation of the mutation in the candidates must be done preferentially. Furthermore, candidates will be used to test, by loss-of-function and gain-of-function lines and after introduction of auxin reporters, the link between PIN3 polarity defects and auxin accumulation. The work is in progress.

ABP1-mediated coordination of cell and tissue polarities in *Arabidopsis*

In multicellular plants, tissue polarization and orientation of polarity axes are central to development. For coordinated polarization, individual cells need to know their position within the tissue and the status of their neighboring cells. How they acquire this information is unclear and conceptually very difficult to imagine, in particularly in plants, where cells are encapsulated within rigid cell walls making cell migration during patterning impossible. One possibility for plant specific polarity establishment is to regulate auxin flow mediated by PIN exporters (Wiśniewska et al., 2006).

In the last part of my work (**Chapter 5**) we reveal an AUXIN BINDING PROTEIN 1 (ABP1)-mediated auxin role important for PIN polarization. Essential for plant growth and development, ABP1 was first characterized as a putative auxin receptor more than 40 years ago in maize (Hertel et al., 1972). An *Arabidopsis* ABP1 null-mutant is embryo lethal (Chen et al., 2001b), which makes it difficult to functionally study. Highly overlooked for a long time, ABP1 became more popular in the last six years, since *abp1* knock-down lines (Braun et al., 2008) were established and bring ABP1 back to the front in the research interest (Sauer and Kleine-Vehn, 2011; Paque et al., 2014; Xu et al., 2014). It has been revealed that ABP1, besides the reported role on cell growth (Chen et al., 2001a), interferes with clathrin-dependent PIN endocytosis (Chen et al., 2012; Robert et al., 2010) and subsequently with PIN polar targeting. Moreover, ABP1 was recently found as negative regulator of the TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALING F-BOX (TIR1/ABF) auxin signaling pathway (Tromas

et al., 2013). TIR1-dependent de-repression of AUXIN RESPONSE FACTOR (ARF) transcription factors results in transcriptional regulation of auxin responsive genes. In this pathway, the F-box protein TIR1 and the closely related AFB proteins act as auxin receptors (Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

We show that ABP1 plays a role in PIN polarity establishment and contributes to PIN polarity re-establishment in developmental processes when auxin flow redirection is required. We have identified cell wall-localized ABP1 as an active central component of polarization processes. We used a combination of *in planta* experiments and computer modelling to reveal the mechanism behind the coordinated behavior of cells in plant tissues. ABP1 feeds back on cell-to-cell transport of auxin thus translating the global auxin gradient across the tissue into polarization of individual cells. This mechanism is capable of propagating polarization from auxin source to sink and generate *de novo* channels of polarized auxin transport, thus providing a basis for downstream tissue patterning. Predictions of the computer model impressively correspond to *in planta* observed cell polarities and auxin distributions in many developmental processes strongly suggesting that the identified mechanism acts largely universally behind polarization processes in plant development.

The missing link to be investigated is the interaction between ABP1 and TIR1 signaling pathways during auxin-mediated PIN polarization. To answer this question we could think of combining loss-of-function mutants in TIR1 signaling pathway (*tir1/afb1,2,3; arf7/arf19* mutants*)* (Sauer et al., 2006) with gain-of-function ABP1 lines which show PIN1 lateralization in the main root regardless auxin treatment (**Chapter 5**). Genetic interaction and interconnection of two signaling pathways would bring the general information about auxin signaling in plants.

Potential agricultural applications of acquired knowledge

Data in this PhD thesis revealed insights into the regulation of gravitropic growth of plant shoot that could be of agricultural interest. Many questions addressed by the regulation of tropic growth in *Arabidopsis thaliana* are of a broader fundamental importance in crops. Therefore, we anticipate that the knowledge gained from our studies in *Arabidopsis* will be of interest for research in crops. The ability of plants to response to the environment is an important determinant of plant fitness. Our research focuses on the basic mechanisms governing plant responses to environment. This mechanism integrates environmental signals such as light, or gravity into reprogramming of plant growth and development. Knowledge into the mechanisms of light and gravity perception regulation is awaited eagerly in the plant field because it addresses one of the central topics of plant development biology: the mechanism underlying adaptive growth in order to increase the fitness of plants in the field in regard to a high field density. This research would lead to manipulations of agriculturally interesting features of plant reactions such as phototropic and gravitropic responses to avoid the shade of neighbouring plants in a plant field.

Over the past few decades, major increases in yield have come largely through increasing field densities. In that case, plants compete for light and nutrients and display typical responses to neighbouring plants. Growth and tropic responses help the plant to position the young leaves to optimize light perception and to overcome the shade imposed by neighbours. One type of these behaviours is shade avoidance (Novoplansky, 2009). It includes elongation of the shoot part, altered flowering time and increased apical dominance (Morelli and Ruberti, 2000). The connection between light-sensing responses (phototropism, photomorphogenesis) and shade avoidance, specifically the role of PIN3 auxin transporter during tropic responses of shoot, and its regulation, is the main target of research (Grebe, 2011; Keuskamp et al., 2010). Here we could consider phototropism as important phenomenon for optimizing light capture, which plays a role together with shade avoidance-induced growth. This research could help to reveal novel tools for specific modifications of plant characteristics in order to interfere with the competition within the plant field. In theory, when the plants in the dense field will not be competing with each other, they could use more energy to the main development and fruit production.

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Summary

The ability to change growth and development of new organs throughout life is a unique feature of plants. Plants are able to regulate cell division and cell elongation and subsequently whole organ growth in response to a changing environment. As endogenous factors, plant hormones largely participate on shaping plant architecture and modulating their growth. One of the hormones regulating most of the plant responses is auxin. Over the last decade, our knowledge increased on how this signaling molecule acts at the level of single cells, different tissues and organs during developmental processes (Kleine-Vehn and Friml, 2008; Vanneste and Friml, 2009).

During its responses to light and gravity, plants accumulate auxin in order to regulate cell elongation of one side of organ and bend according to the stimuli direction. Regulation of cellular plasma membrane polarity of auxin efflux carriers is of high importance during this process (Friml et al., 2002). PIN3 auxin efflux carriers change their plasma membrane localization in order to regulate auxin flow and play a major role in tropic responses in root and hypocotyl cells (Kleine-Vehn et al., 2010; Rakusová et al., 2011). Here in Chapter 2 I present the intracellular regulation of PIN3 relocation during gravitropism.

In this PhD thesis (Chapter 3), I reveal a novel mechanism of regulating PIN3 localization, which is feed-back regulated by auxin itself and used as a mechanism to prevent over-bending of the hypocotyl. The mechanism by which auxin mediates PIN3 inner-lateralization is based on PIN3 protein abundance within the cell. This finding highlights a so far unknown mechanism of how plants regulate their response to the environment.

We demonstrate that both processes could be compared by a reverse genetic approach. PIN3 polarization is regulated at several levels of intracellular trafficking. To characterize the unknown components, I employed a forward genetic strategy. I obtained several candidates with different defects in PIN3 polarization in hypocotyl endodermal cells. I plan to further characterize them in future. Progress on this project is described in Chapter 4.

Auxin feeds back on its own transport by regulating polar localization of PINs during plant development (Sauer et al, 2006). Our study identified the mechanism underlying coordinated cell and tissue polarization during various developmental processes in plants. The classical example of this auxin canalization during the plant development is embryogenesis, lateral root development and vasculature development. The PIN polarizing signal is controlled by the nuclear TIR1 (Sauer et al, 2006) and the recently revisited extracellular ABP1-mediated

auxin signaling machinery. Via such a mechanism auxin feeds back on its directional movement between cells. We present a model capable to generate coordinated emergence of cell polarities within non-polarized tissue. The model simulations reproduce known patterns of polarity and auxin-based patterning as well as make important predictions in terms of auxin effect on polarity and various requirements of both nuclear and extracellular auxin perception mechanisms. In line with the central role of ABP1 in our polarization mechanism, experiments revealed a key role of ABP1 in cell polarization processes during developmental processes such as organogenesis or vascular tissue formation. Data presented in this PhD thesis (Chapter 5) support the extracellular ABP1-mediated feed-back on auxin transport as a central, plant-specific molecular framework for the coordination of cell and tissue polarity patterns during plant development.

Altogether, this PhD research brings novel insights into the mechanisms of the auxin role during plant development and plant environmental responses. We gain more insight into ABP1-mediated auxin signaling and its contribution to cell polarization, cell growth and subsequently plant development. We aim to gain novel knowledge by focusing on the mechanisms underlying regulation of cell polarity in plants by external signals. We believe that this work will allow us to understand how this individual cell-based signalling is translated into morphogenetic auxin gradients mediating growth decision at the level of a multi-cellular plant organism.

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(*joint first)

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- **Rakusová H**, Robert HS**,** Friml J; Auxin feed-back on PIN3 polarity for termination of shoot tropic responses.

- Rakusová H*, Wabnik K*, Sauer M, Grones P, Barbez E, Kaufmann WA, Rycke R, Chen X, Simon S, Robert SH, Van Montagu M, Kleine-Vehn J, Friml J; ABP1-mediated coordination of cell and tissue polarities in *Arabidopsis*.

- Chen X, Grones P, Rycke R, Chen J, Wabnik K, Rybel B, Lin D, **Rakusová H**, Cannoot B, Weijers D, Perrot-Rechenmann C, Yang Z, Friml J; Auxin mediates ABP1 secretion through SKU5 and SKS interacting partners in *Arabidopsis.*

Wabnik K[!], **Rakusová H**, Rycke R, Friml J; Auxin flux sensing via receptor clustering predicts coordinated polarization in plants.

(*joint first; [!]corresponding)

Presentation

Auxin feed-back on PIN3 polarity prevents gravitropic overbending in *Arabidopsis* hypocotyl. **Auxin cytokinin meeting**, Prague/ Czech Republic, 2014.

Mechanisms of auxin feed-back on PIN3 polarity for hypocotyl tropism in *Arabidopsis.* **The 8th SPPS PhD Students Conference**, Uppsala/ Sweden, 2014.

Poster presentation

Identification of gravitropism-related PIN3 polarity regulators in *Arabidopsis***. International conference on Arabidopsis Research (ICAR)**, Vancouver/ Canada, 2014.

Unravelling mechanism of auxin mediated PIN3 re-location in *Arabidopsis* hypocotyls. **16th ENPER meeting**, Gent/ Belgium, 2013.

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